PROTEIN-CELLULOSE INTERACTIONS IN THE SPORE COAT OF *DICTYOSTELIUM DISCOIDEUM*

BY

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PROTEIN-CELLULOSE INTERACTIONS IN THE SPORE COAT OF *DICTYOSTELIUM DISCOIDEUM*

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The spore coat of *Dictyostelium,* consisting mainly of protein and cellulose, has been used to study protein-cellulose interactions. Proteins extracted from the cellulose shell of spore coats were precipitated specifically by cellulose but not Sephadex or Sepharose *in vitro.* Among the nine major proteins, SP85 and SP35 were found to be good candidates for direct cellulose-binding whereas SP65 bound Avicel only in the presence of SP85. A β-glucosidase from the adjacent interspore matrix was found to bind cellulose by a distinctive mechanism.

Edman sequencing, amino acid analysis and Western blotting showed that SP85 and SP35 are encoded by the *psp*B and *psv*A genes, respectively. SP35 is equivalent to the N-terminal cysteine-rich domain of the *psv*A gene product, probably derived from the previously detected p58 by proteolytic processing. PCR sequencing of *psp*B genomic DNA suggested that SP85 is a modular protein consisting of 3 domains separated by tandem TXPP repeats. Expression of partial cDNAs of SP85 in *E. coli* confirmed that SP85 has intrinsic cellulose-binding activity, and localized this activity to the cysteine-rich C-terminal domain which also mediated binding to SP65.

The relevance of these *in vitro* observations to spore coat properties *in vivo* was tested using genetic approaches. Expression of portions of SP85 in prespore cells showed that the C-terminus of SP85 is responsible for its coat incorporation, consistent with the cellulose-binding properties of this domain *in vitro*. Deletion of SP85 by gene replacement of *psp*B resulted in a failure of SP65 to incorporate, which could be rescued by expression of the C-terminus or a fusion of the N- and C-termini. Deletion of SP85 or overexpression of its individual domains resulted in negative effects on coat barrier functions, as inferred from increases in coat permeability to a macromolecule tracer and the efficiency of spore germination. Together, the data suggest that SP85 contributes in an essential fashion to barrier functions of the coat as a cross-linker, by virtue of individual domains which mediate specific protein-cellulose and protein-protein interactions.

CHAPTER 1
BACKGROUND AND INTRODUCTION

Extracellular Matrix (ECM)

Biological Significance of Extracellular Matrix

Extracellular matrix (ECM) is made of molecules secreted by all types of prokaryotic and eukaryotic cells either in unicellular or multicellular organisms. Different types of organisms and their tissues have different forms of extracellular matrix. A relatively uniform two-dimensional cell wall surrounds individual cells of bacteria, fungi and plants. In animals, however, the ECMs become rather complicated, each specialized for a particular function such as strength in tendon or filtration in glomerulus of kidney. Most ECMs in animal tissues have no delineated boundaries between individual cells, such as those surrounding fibroblast cells in loose connective tissue and hence are three dimensional. Nevertheless, several special ones, such as basement membrane under epithelia cells separating tissue compartments, and zona pellucida on the surface of an ovum, are morphologically similar to two-dimensional cell walls.

The ECM not only physically supports and separates individual cells from the external world, but is also involved in a variety of vital functions. It provides a selective permeability barrier to the enclosed cell and substrate for binding of enzymes (Basco et al., 1993; Chavakis et al., 1998), hormones and growth factors (Massague, 1991; Yayon et al., 1991). In multicellular organisms, the ECM also plays instructive roles in morphogenesis by regulating cell shape (Varner, 1989; Cosgrove, 1997), providing substrates for cell migration (Linask et al., 1988), cell-cell interaction (Hinsch et al., 1997) and induction of cell differentiation (Knezevic et al., 1998). In summary, the ECM has come to be recognized as an important vital organelle rather than an inert exoskeleton (Stratford, 1994; Cid et al., 1995; Cosgrove, 1997).

Function of ECM can be impaired if one or more components are genetically or biochemically altered, thus resulting in disease. For example, mutation of laminin 5, which is a component of epidermal basement membrane, can result in epidermolysis bullosa (Uitto, 1996; Takizawa et al., 1998). Overproduction of renal extracellular matrix components causes malfunction of the kidney, a symptom of insulin-independent diabetes mellitus (Ziyadeh, 1995; Wu et al., 1997). ECMs are also involved in attack or defense actions between host and pathogenic microorganisms. Pathogens can bind ECM components of host cells, which is often an essential intermediate step to pathogenesis (Conway and Ronald, 1988). The host cell, on the other hand, may release chemicals into ECM to destroy the intruder (Stintzi et al., 1993).

Although knowledge about ECM is increasing, much remains to be explored about specific molecular interactions within the ECM. The understanding of these specific interactions is essential for understanding the roles of ECM in normal or pathogenic processes. Consequently, for the benefit of human beings, we could devise strategies to restore disrupted barrier functions or to prevent pathogenesis of microorganisms.

Mechanisms Involved in the Formation of ECM

Polysaccharide and protein are two major types of molecules in all kinds of ECM, but the type of polysaccharide and protein and the ratio between the two differs among different organisms and their tissues. For instance, in the yeast cells, (1→3)-β-glucan and chitin (poly-[1→4]-N-acetyl-D-glucosamine) compose up to 75% of the wall mass (Barran et al., 1975; Stratford, 1994); in the cell walls of *dicotyledon*ous plant, cellulose (a linear β [1→4] glucose polymer), hemicellulose (a branched [1→4]-β-glucan containing xylose, galatose and fucose) and pectin (an acidic heterogeneous polysaccharide) each composes ~30% of the wall with as little as 1-5% of protein (Cosgrove, 1997). In comparison, spore coats of bacteria (Hills, 1973) and ECMs of animals are richer in protein than polysaccharide (Leblond and Inoue, 1989).

Though the composition and structure of ECMs differ from one organism to another, the general idea is that a diverse array of molecules interact to form an ordered and layered multi-dimensional structure (Leblond and Inoue, 1989; West and Erdos, 1990; Aronson et al., 1992; Cid et al., 1995). Various models of ECM formation have been proposed, which can be summarized in two categories correlating the relative mass ratio of protein to polysaccharide. In fungal and plant cells, carbohydrate polymers are proposed to form a fibril framework, in which proteins, in relatively small percentage, fill as "pebbles in a sand/cement matrix" (Elorza et al., 1993; Stratford, 1994). In contrast, proteins, the major components of the ECMs of animals and bacteria spores, are thought to self-assemble to form a proteinacious fibril network, in which polysaccharides and other proteins fill. For example, collagen type IV and laminin, the major protein types in the basement membrane, have been found to self-assemble *in vitro* to form microfibrils as seen *in vivo* (Leblond and Inoue, 1989). CotE and CotT proteins are essential to form a fibril backbone in the spore coat of *Bacillus subtilis* (Aronson et al., 1992). Similarly, proteins extracted from the *Chlamydomonas reinhardtii* cell wall were able to reconstitute a wall-like structure after dialysis to remove a chaotropic regent (Catt et al., 1978; Hills, 1973). Both of the two models described above may apply to the spore coat of *Dictyostelium,* which consisted of half protein and half cellulose (West et al., 1996; Table 1-1). Proteins were postulated to have minor structural roles in carbohydrate polymer-rich walls mainly because of their relatively low abundance, which both slowed the effort to explore their actual role. In *Dictyostelium,* various biochemical and genetic tools are available to study the role of coat proteins.

The overall structure of ECMs is probably initiated by noncovalent interactions and reinforced by covalent interactions among components (Keegstra et al., 1973; Fleet, 1991; Elorza et al., 1993; Lu et al., 1995; Hauser et al., 1996). Noncovalent interactions often involve one individual domain of a modular protein as discussed below. Protein and polysaccharide are often cross-linked through glycosyl bonds (Lu et al., 1995; Kapteyn et al., 1997). Common covalent bonds between proteins include those catalyzed by transglutaminase (Schittny et al., 1997), lysyl oxidase (Rucker et al., 1998), peroxidase of isodityrosine (Brady et al., 1998) and disulfide isomerase (Wilson et al., 1998). Most of the covalent bonds are irreversible except disulfide bonds which can be reversed with a reducing agent. The study of direct interactions in ECM is often slowed by the irreversible covalent cross-linkages, the limited material and the effort to do genetics in complex systems (Cooper and Varner, 1983; Henriques et al., 1998; Evans et al., 1990; De Nobel and Lipke, 1994). The spore coat of *Dictyostelium*, rich in disulfide bonds, but not other covalent bonds (West et al., 1996), promises to be a suitable model system to explore the role of protein-protein and protein-polysaccharide interactions in the formation of ECM at a molecular level.

Modular Nature of ECM Proteins

A common feature of structural proteins is that they are modular, consisting of multiple globular and rod-like domains with autonomous activity (Engvall and Wewer, 1996). Besides catalysis, three common functions of domains are as rubber bands, spacers and binding to other components, which are represented by fibronectin type III (Oberhauser et al., 1998); serine/threonine mucin-like repeats (Tsiang et al., 1992) and the EGF-like module (Fehon et al., 1990), respectively. The rubber band domains, for example in elastin, contribute elasticity to ECM. Spacers are probably essential for spatial separation and presentation of attached domains. The specific interactions among a diverse array of mosaic proteins contribute to the complexity and integrity of ECM (Engel, 1996; Hegyi and Bork, 1997). Spore coat proteins of *Dictyostelium*, similar to mammalian structural proteins, contain modular repeats, most commonly cysteine rich EGF-like and serine/threonine rich mucin-like repeats (Haberstroh et al., 1991a; West et al., 1996). It is possible that the cysteine rich domains are important for protein coat assembly through interactions with cellulose or other proteins.

Spore Coat of *Dictyostelium Discoideum*

The Spore Coat of *Dictyostelium Discoidum* as a Model System for Study of ECM

Development of *Dictyostelium* *Dictyostelium discoidum* is a primitive eukaryote which diverged around the separation of plant and animal lineages in the evolutionary tree (Loomis and Smith, 1995). The development of *Dictyostelium discoidum* is separate from its growth. Cells grow and divide by typical mitosis as individual cells when supplied with sufficient food. When the food is depleted, up to a hundred thousand cells aggregate in a streaming pattern and start migration as a slug. During migration, cells differentiate into either prespore cells or prestalk cells. In response to environmental cues, prespore cells rise on top of prestalk cells to form the fruiting body that contains 80% spores and 20% stalk cells. It takes about 24 hours for vegetative growing cells to differentiate and form a mature fruiting body. The stalk cells are vacuolated and dead. Each spore produces *de novo* a semipermeable (Cotter, 1981), distensible (Lydan and Cotter, 1994) and protective (Kessin et al., 1996) coat on its surface. Thus, the enclosed amoebae are resistant to harsh environments, such as dehydration, starvation, temperature extremes and toxic chemicals. Under appropriate conditions, spores germinate and continue the life cycle as vegetative growing cells (Loomis, 1975).

ECMs made during development of *Dictyostelium* In addition to the spore coat, at least four types of ECM are made during development of *Dictyostelium.* A type of ECM, which facilitates cell movement (Williams and Joss, 1993), is secreted by early stage aggregating cells. During migration of the slug, a slime sheath is continuously made on the surface of the whole migrating slug and deposited on the substratum as a trail marking the slug's progress (Blanton, 1993). There is also an interstalk matrix between stalk cells and an interspore matrix between spores. The interstalk matrix and the slime sheath are probably made by prestalk cells and have similar biochemical components including cellulose and two proteins with tandem sequence repeats, ECMA and ECMB (Blanton, 1993; McRobbie et al., 1988). Although different in protein composition, all matrices contain cellulose and may share similar mechanisms of formation (West and Erdos, 1990). Protein differences may explain the different organization and functions of these matrices. This study was focused on the spore coat for reasons discussed below.

The spore coat of *Dictyostelium* as a model system for study of ECM There are several advantages of the spore coat that allow study of the mechanism of ECM formation at both biochemical and molecular levels. First, the spore coats can be purified to a high degree of homogeneity in contrast to, for example, the mammalian basement membrane. Second, there are no covalent linkages other than disulfide bonds between the major components of spore coats, protein and cellulose (Wilkinson and Hames, 1983; West et al., 1996), which allows us to explore individual molecules involved in protein-protein and protein-cellulose interactions without using proteases. Third, the *Dictyostelium* system is suitable for molecular biology manipulation by both targeted gene disruption (Kuspa and Loomis, 1994; Fosnaugh et al., 1994) and stable inducible gene overexpression (Rebstein et al., 1993; Blusch et al., 1992; Ramalingam et al., 1992). Furthermore, the formation of the spore coat is independent of cell growth so that mutants defective in coat formation are expected to be viable. In summary, the

Dictyostelium spore coat is a favorable system to study the role of interactions between protein and cellulose polysaccharide in the formation and function of extracellular matrix.

Composition and Structure of Spore Coat

Each spore coat contains about 500 fgs of nine major proteins (Table 1-1), 330 fgs of cellulose and less than 50 fgs of garulan (White et al., 1963; Aparicio et al., 1990; West et al., 1996). By heavy metal staining of thin sections, the spore coat appears as a three-layered structure consisting of two electron dense layers, 30 nm each in thickness, separated by a 150 nm thick electron lucent layer (Hemmes et al., 1972). The two electron dense layers are shown to be proteinaceous as assayed by protease accessibility and electron microscopy after immuno-gold labeling. Epitopes of five out of nine major coat proteins have been located in the outer layer (Table 1-1). In comparison, SP85, galactose, GalNAc residues and several lysosomal enzymes are located in the inner layer adjacent to the plasma membrane (Devine et al., 1983; Erdos and West, 1989; Lenhard et al., 1989; West and Erdos, 1988, 1992; Watson et al., 1994; Aparicio et al., 1990). The middle layer is primarily cellulose, which is identified by cellulase-gold labeling (Erdos and West, 1989) and sensitivity to cellulase digestion (Hemmes et al., 1972). Freeze fracture/etch replicas reveal two sublayers of microfibrils. The microfibrils in the inner and outer sublayers are oriented randomly and transversely, respectively, to the long axis of spores (Hemmes et al., 1972). The elaborate structure and the distinctive localization of components indicate that the assembly of the spore coat is a regulated process.

Assembly of Spore Coat

Assembly of spore coats occurs in two phases (Figure 1-1). Coat proteins and galurans are synthesized in the rER and stored in post-Golgi prespore vesicles (Hemmes et al., 1972; Devine et al., 1983). As the prespore cells rise up on stalk cells during morphogenesis, coat proteins and galurans are exocytosed and accumulate amorphously between spores (West and Erdos, 1992). Most of the proteins are diffusive and exchanged between neighboring spores. Subsequently, cellulose emerges presumably from the plasma membrane as in stalk cells and other systems (Blanton et al., 1990; Blanton, 1993; Grimson et al., 1996; Haigler et al., 1996). Coincident with the presence of cellulose, proteins segregate into either an outer or inner electron dense layers (Erdos and West, 1989). The overall structure of the spore coat is asymmetrical from outer layer to inner layer adjacent to the plasma membrane. Studies from other organisms have found two mechanisms that could contribute to the polarity of structure, including sequential secretion of ECM components and environmental asymmetry imposed by the external world and the plasma membrane. For example, the components of the outer primary wall are secreted and deposited before those of the inner secondary wall in plant cells (Aronson et al., 1992, Carpita et al., 1993). The plasma membrane is an anchor for inner layer components such as GPI-linked proteins in yeast (Vai et al., 1992) and integrin receptors in basement membrane (Clark, 1990; Ekblom, 1996). In the spore coat of *D*ictyostelium, however, outer and inner layer proteins were secreted at approximately the same time. It suggests that novel types of mechanisms are probably significant in the final structure of spore coat, such as interactions between protein and cellulose.

Cellulose is synthesized from UDP-glucose monomer, presumably by a protein complex on the plasma membrane (Blanton et al., 1993) as a bundle of polymers. The nascent polymers, 1.5 nm thick, further polymerize and crystallize in a parallel fashion to form up to 25 nm diameter microfibrils (Haigler and Benziman, 1982). The parallel assembly of cellulose has been shown in other systems to be energetically unfavorable and to require specific enzymes (Gardner, 1978; Haigler and Benziman, 1982; Saxena et al., 1994). In the *Dictyostelium* spore coat, the presecreted proteins may provide an important milieu for polymerization and crystallization of cellulose fibrils.

The importance of proteins and polysaccharide in the coat has been investigated by genetic deletion of specific components. In *mod*B and *mod*C glycosylation mutants, spores tend to have reduced viability after aging and the coats became more permeable to a macromolecular tracer (Aparicio et al., 1990; Gonzalez-Yanes et al., 1989). Null mutations of three outer layer coat proteins, including SP96, SP70 and SP60, collectively resulted in the increase of the coat permeability (Fosnaugh et al., 1994). These data indicated that both glycosylation and outer layer coat proteins contribute to the barrier functions of the coat. Mutants in UDP-glucose phosphorylase 2 (UDPGPP2), an enzyme essential for the synthesis of cellulose (Dimond et al., 1976), fail to form spores. Immuno-colocalization has suggested that glycoprotein complexes interact with cellulose in the slime sheath (Zhou-Chou et al., 1995). Similarly, cellulose and protein may interact with each other in the spore coat. Our goal in this project is to find the hypothetical cellulose-binding proteins *in vitro* and study their function *in vivo*.

Disassembly of Spore Coat

Partial disassembly of spore coat happens during germination. Once stimulated by heat (42 °C), or small molecules such as urea, amino acid or peptone (Cotter, 1968), spores swell with the outer and middle layers being ruptured (Cotter and Rapper, 1968; Cotter et al., 1969). During this process, activities of cellulases and proteases are detected (Jones et al., 1979; Blume and Ennis, 1991). During emergence, the amoeba squeezes through a split, leaving the rest of the coat almost physically intact.

Spores can also be forced to open mechanically by high pressure (20,000 psi) in a French pressure cell. Coats purified from either post-germination or pre-germination by gradient centrifugation to homogeneity (see Fig 2-1) can be disassembled biochemically *in vitro* by hot urea and 5%-β-ME. The soluble urea extract includes more than 90% of coat proteins, indicating that the interactions between protein and cellulose are not covalent excluding disulfide bonds (Wilkinson and Hamme, 1983; West et al., 1996).

Interspore Matrix

Spores are held together by surface tension on top of stalk cells as an aqueous cell suspension. The material between spores, the fluid interspore matrix (ISM) bordering the outer layer of spore coat, is readily solubilized in aqueous solution. Several glycoantigens, including GA-XXXII and GA-XXX, recognized by mAb 81.8 and 85.2, respectively, have been found to accumulate uniquely in the interspore matrix (West and Erdos, 1988). The composition of major proteins in the ISM is different from that of coats. Nevertheless, several spore coat glycoproteins, including SP75, SP85, SP86, SP87 and SP96 were detected, albeit at low levels, in this space (West and Erdos, 1990, 1992, 1995; Yoder et al., 1994). These findings suggest that the ISM may provide a good source of unassembled native spore coat proteins which do not require exposure to denaturants for solubilization. Using the cellulose-binding assay to test for cellulose-binding proteins in the ISM revealed that the enzyme, β-glucosidase-2, has cellulose-binding activity, probably with a different mechanism compared to coat proteins. This finding suggested that the ISM is not merely a default reservoir for unused coat components.

Conclusion

Each ECM is composed of a diverse array of molecules through specific molecular interactions, which is the essential and common theme for the formation and function of ordered ECM structures. It is necessary to understand these specific interactions to fully address the variety of functional and pathogenic issues of ECM. The spore coat of *Dictyostelium* is chosen as a suitable ECM system to study protein-protein and protein-cellulose interactions due to its relative simplicity and accessibility to genetic manipulations. This is shown in the following chapters, where cellulose-binding proteins were studied using *in vitro* biochemical and *in vivo* genetic approaches.

Table 1. The major components of the *Dictyostelium* spore coat[1 & 4]

Proteins	Gene	Mutant strain	Probes	Layer at which probe localizes[1]
SP96	*cot*A	TL55, TL56[2]	mAb 83.5, anti-SP96	outer
SP87	*psp*D (PL3)		anti-PL3	middle/outer
SP85	*psp*B (14E6)	B^{-}[4]	mAb 16.1, MUD102	inner/middle
SP75	*cot*D (DP87)	DP87^{-}[3]	anti-DP87, mAb 83.5	outer
SP70	*cot*B	TL26, TL56[2]	anti-SP70	n.t.= not tested
SP65			none	n.t.
SP60	*cot*C	TL52, TL56[2]	anti-SP96	n.t.
SP52			none	n.t.
SP35	*psv*A		anti-p58(C), anti-p58(H)	n.t.
Polysaccharides				
cellulose			cellulase	middle
galuran			RCA-1	inner

1. West and Erdos, 1990; West et al., 1996; Yoder et al., 1994.
2. Fosnaugh et al., 1995
3. Nakao et al., 1994
4. Chapter 4 in this study.

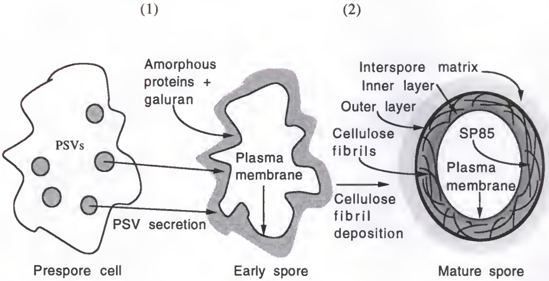


Figure 1-1.Two phases of *Dictyostelium* spore coat formation. 1. Secretion of coat proteins and garulan from prespore vesicles. 2. Cellulose fibril deposition and organization of coat components into three layers. Some species of the coat components fail to incorporate into the coat and accumulate in the interspore matrix.

CHAPTER 2
TWO PROTEINS OF THE *DICTYOSTELIUM* SPORE COAT BIND TO CELLULOSE *IN VITRO*

Introduction

Formation of the *Dictyostelium* spore coat begins with the secretion of most, if not all, of its future proteins and galuran, which are pre-made in rER and stored together in prespore vesicles (Hemmes et al., 1972; Devine et al., 1983; West and Erdos, 1990). Co-immunoprecipitation studies have suggested that some of the proteins form a soluble complex before secretion (Devine et al., 1983; Watson et al., 1993, 1994; McGuire and Alexander, 1996). After secretion, the distribution of most of the proteins is unlocalized between neighboring spores until cellulose, emanating from the plasma membrane, is deposited. After the appearance of cellulose, specific protein domains are found either external to, or internal to and partially overlapping with the cellulose layer, as suggested by sensitivity to proteases and immunogold labeling (Orlowski and Loomis, 1979; Erdos and West, 1989; West and Erdos, 1988, 1990; West et al., 1996). These observations imply that coat protein complexes do not become incorporated into a coat structure until cellulose appears. Furthermore, coat proteins extracted from cellulose remain soluble after removal of denaturants and reducing agents by dialysis or gel filtration (West et al., 1996), which suggest that protein-protein interaction is not enough to form an insoluble matrix. It is possible that protein-cellulose interactions initiate coat assembly by mutually trapping proteins in the coat and guiding cellulose morphogenesis.

To investigate the interaction between protein and cellulose, coat proteins were selectively extracted from coats and reconstituted with Avicel (a type of microcrystalline cellulose), Sephadex or Sepharose in microcentrifugation tubes. It was found that more than half of the protein extracts bound to cellulose but negligible amount of protein bound to Sephadex or Sepharose. However, when the proteins were partially separated by ionic exchange columns, only two proteins were able to bind to cellulose as a single enriched protein in the fraction. These two proteins, SP35 and SP85, were encoded by previously reported *psv*A and *psp*B genes, respectively. SP35 may be derived proteolytically from the full-length product of *psv*A, which was previously shown missing in the mature spore coat. Sequencing of the PCR product from genomic *psp*B DNA showed that the published *psp*B gene incomplete and should include an intron and a TXPP repeat coding region with a C4C repeat embedded among them. Expression of partial cDNAs of SP85 confirmed its intrinsic cellulose-binding activity and localized this activity on its C-terminus. These findings suggest a model by which coat proteins associate with cellulose via direct or indirect specific interactions.

Material and Methods

Cell Manipulation

Wild-type strains X22, WS380B, WS576, and NC-4 were passaged on lawns of *Klebsiella aerogenes* grown on SM agar plates. The axenic strain Ax3 was grown on HL-5 medium (West et al., 1996). Spores were allowed to form after consumption of the bacteria, and were collected at <1 day of age. For germination, spores were transferred to the lid by slapping the inverted plate onto a counter top, and the spores were recovered by jetting H_2O onto the surface with a Pasteur pipette and collecting the suspension into a polypropylene tube, as described (West et al., 1996). Contamination by stalks was reduced by vacuum filtration through a 120-mesh steel screen, and spores were recovered by centrifugation at 2000 rpm for 1 min. The supernatant was saved as the interspore matrix fraction.

Purification of Spore Coats and Extraction with Urea

Coats were isolated either from post-germination cultures or directly from intact, i.e., pre-germination spores. For germination, spores from 100 10 cm plates were suspended at 5 x 10^7 ml^{-1} in KP buffer (10 mM potassium phosphate, pH 6.5), activated at 42 °C. for 30 min, and diluted to 2 x 10^7 ml^{-1} with KP buffer for germination on a rotary shaker overnight at 22 °C. Coats were purified by differential and density gradient centrifugation as described by West (West et al., 1996). The culture supernatant was saved as the germination supernatant. For isolation of coats from pre-germination spores, spores from 400 plates were suspended at 7.5 x 10^8 ml^{-1} in KP, and lysed at 5 °C in a French pressure cell at 20,000 psi. Coats were centrifuged at 9500 g for 5 min, and resuspended in KP with or without 0.1% (v/v) NP-40 for purification as described above. After purification, coats were extracted with 8 M urea, 20 mM Tris-HCl, pH 7.4, with 5% (v/v) 2-ME, 1 mM phenylmethylsulfonyl fluoride, 10 μg leupeptin ml^{-1}, and 18.5 μg aprotinin ml^{-1}, for 3 min in a boiling water bath. Extracted proteins and residual coats were separated by centrifugation at 18 x 10^3 g for 20 min. In some cases, the urea extract (supernatant) was dialyzed using cellulose ester membrane with a molecular weight cut-off of 10 x 10^3 (Spectrapor) against 50 mM NH4Ac, pH 7.4, or desalted over a PD10 cartridge (Pharmacia, Piscataway, N. J.), which contained 9 ml of Sephadex G-25 equilibrated in 50 mM NH4Ac, pH 7.4. Soluble protein concentration was estimated by a commercial Coomassie Blue dye-binding assay (Pierce), with BSA as a standard.

Monosaccharide Composition Analysis

Samples were hydrolyzed either by incubation in 0.5 ml 4 M trifluoroacetic acid for 4 h at 100 °C (mild acid hydrolysis), or by heating for 1 h in 0.5 ml 100% trifluoroacetic acid, followed by dilution with an equal volume of water and additional incubation at 100 °C for 3 h (strong acid hydrolysis). Mild hydrolysis conditions were typical for monosaccharide analysis of glycoproteins (Kozarov et al., 1995), and the strong hydrolysis conditions were those used for wood cellulose (Fengel and Wegner, 1997). Samples were then taken to dryness in a vacuum centrifuge and reconstituted in 17 mM NaOH. Sugars were analyzed on a Dionex PA-1 column by high pH anion-exchange chromatography (Kozarov et al., 1995), using a Dionex ED-40 pulsed amperometric detector.

Ion Exchange Chromatography

Urea extracts were applied at 22 °C to a TSK DEAE-5PW (8 x 75 mm) column (Toyo Soda, TosoHaas, PA) on an LKB GTi FPLC system at 0.75 ml min^{-1}, pre-equilibrated in 50 mM NH_4Ac, pH 6.0, with or without 6 M urea as indicated. The column was typically eluted with a gradient of 50 mM to 1.5 M NH_4Ac, pH 6.0. The DEAE flow-through fraction of some trials was supplemented with PMSF, leupeptin and aprotinin as above, adjusted to pH 5.0 with H_3PO_4, and applied at 22 °C to a TSK SP-5PW (5 x 5 mm) column (Toyo Soda) at 0.5-0.75 ml min^{-1}, pre-equilibrated in 6 M urea, 20 mM NaH_2PO_4, pH 5.0. The column was eluted with a gradient from 0 to 1 M NH_4Ac in the same solution, then equilibrated in 4 M urea, 0.25 M NH_4Ac, 20 mM sodium phosphate, pH 5.0, and eluted further at 0.25 ml min^{-1} with a second gradient from pH 5.0 to pH 8.0 in the same solution. 0.5 ml fractions were collected. Ion exchange columns were regenerated after each use by successive treatment with 0.1 N NaOH and 30% HAc.

Avicel Column Chromatography

The DEAE flow-through fraction from pre-germination coats was concentrated in a Centricon-10 centrifugal ultrafiltration device (Amicon, Waverly, MA) and desalted on a PD-10 cartridge equilibrated in water to remove urea and 2-ME. Hydrated Avicel (Type PH-101, FMC Corporation, Philadelphia, PA) was de-fined, loaded into columns (1 ml bed volume), and equilibrated in 50 mM NH4Ac, pH 6.8. Protein in 5-10 ml volume was applied in a rate of 5-10 ml h^{-1} at 22 °C, and eluted under gravity flow at 1-2ml min^{-1} sequentially with 5 ml each of 50 mM NH4Ac, pH 6.8; H_2O; 8 M urea, 50 mM NH4Ac, pH 6.8; and 2% (w/v) SDS, 50 mM NH4Ac, pH 6.8; each eluent contained 5% (v/v) 2-ME. The flow rate was controlled by adjusting the vertical distance between of the sample outlet tubing opening and the Avicel bed.

Centrifugal Cellulose-Binding Assay

Urea extracts were desalted either by gel filtration or dialysis (see above). DEAE-fractions were diluted in H_2O so that the NH4Ac concentration was ≤0.1 M. SP-column fractions were concentrated in a Microcon-30 centrifugal ultrafiltration cartridge (Amicon) down to 20-180 µl, and diluted 50-200 fold with 50 mM NH4Ac, pH 6.8. 10-70 µl of the protein sample to be tested, and 300-3000 µg of Avicel, from a 100 mg/ml stock suspension in 50 mM NH4Ac, pH 6.8, were brought to a final volume of 120 µl with 50 mM NH4Ac, pH 6.8, and incubated in a 1.5 ml polypropylene microcentrifuge tube on a gyratory shaker for 16 h at 22 °C. After centrifugation at 10,000 *g* for 1 min, the non-bound fraction was removed with a pipette, and the pelleted bound fraction was washed dispersed and repelleted 3 x with 50 mM NH4Ac, pH 6.8. The pellet was resuspended in SDS-PAGE sample loading buffer with 5 mM DTT, boiled for 3 min, and centrifuged again. Equivalent proportions of the non-bound and bound (SDS-released) fractions were compared by SDS-PAGE.

Electrophoresis, Western Blotting, Microsequencing and Composition Analysis

Samples were dried in a vacuum centrifuge to reduce volatile salts, and dissolved in the SDS-PAGE loading buffer (62 mM Tris-HCl, pH 7, 2% (w/v) SDS, 10% (v/v) glycerol) by boiling for 3 min. Electrophoresis was performed on 7-15% linear gradient SDS-polyacrylamide gels followed by staining with Coomassie Brilliant Blue R-250. For Western blotting, gels were electrotransferred to 0.45 μm pore diameter nitrocellulose membranes (Schleicher & Scheull), or Immobilon P^{SQ} PVDF membranes (Millipore), in a BioRad TransBlot SD semi-dry transfer cell. The top buffer was 50 mM glycine, 50 mM Tris-HCl, pH 8.5, and 0.1% (w/v) SDS; the bottom buffer was 50 mM glycine, 50 mM Tris-HCl, pH 8.5, prepared in 20% (v/v) MeOH. For antibody probing, nitrocellulose membranes were incubated in primary antibody diluted in 5% nonfat dry milk, followed by alkaline phosphatase-conjugated secondary antibodies and colorimetric detection as described (West et al., 1996). For Edman degradation studies, samples were first boiled at 100 °C for 3 min in SDS-PAGE sample buffer with 50 mM DTT, followed by addition of iodoacetamide to 100 mM and incubation in the dark for 1 h at 22 °C. The SDS-PAGE gel was prerun in the presence of 10 mM sodium thioglycolate in the upper buffer for 30 min before applying samples (Dunbar et al., 1994). Proteins blotted to PVDF membrane were visualized by Coomassie Blue staining. Corresponding PVDF membrane fragments were introduced onto an ABI model 494 Procise sequenator. For internal sequencing, the stained gel band was partially dried, rehydrated with 20 mg ml^{-1} CNBr in 70% formic acid, macerated, incubated at 37 °C in the dark for 16 h, and then subjected again to SDS-PAGE using a Tris-tricine buffer system (Ploug et al., 1989), followed by electroblotting.

For amino acid composition analysis, protein prepared as for Edman degradation was hydrolyzed in 6 N HCl with norleucine as an internal standard. PTC derivatives were prepared and analyzed as described (Ozols, 1990).

Viscometric Cellulase Assay

Extracts and column fractions were incubated with calibrated volumes of carboxymethylcellulose at pH 5.0 at 37 ˚C, as described (Shoemaker and Brown, 1978). Less activity was detected at pH 7.0. The time required to drain 1 ml of the solution was determined in second. Shorter drain times correlate with decreased viscosity of the carboxymethylcellulose solution and increased endoglucanase activity.

In Situ Cellulase Assay in Gels

Extracts and column fractions were subjected to SDS-PAGE as described above, except that samples were heated at 37 ˚C, as a previous trial of solubilization at 100 ˚C resulted in much lower activity. After electrophoresis at 22 ˚C, gels were washed and equilibrated in 50 mM NaH_2PO_4, pH 5.0, for 3 x 10 min. Cellulase activity was detected by incubating the gel in 1% (w/v) carboxymethylcellulose in 50 mM potassium phosphate, pH 5.0, for 2 h at 37 ˚C, rinsing the gel with 50 mM potassium phosphate, pH 5.0, twice for 10 min each, and staining with 1% (w/v) Congo Red in the same buffer for 20 min, followed by destaining with 1 M NaCl until bands were clearly revealed, as described (Mathew and Rao, 1992). Gels were photographed through a type 40A Kodak Wratten filter. Proteins were non-specifically stained by Congo Red, and cellulase activity was detected as regions of negative staining.

DNA Analysis by PCR from Genomic DNA

pG219 and p14E6Pst73#1, which contained *psp*B DNA and cDNA, respectively, (Powell-Coffman and Firtel, 1994) were generously provided by R. A. Firtel and J. Coffman-Powell and amplified in *E.coli* strain DH5a. For genomic DNA preparation, 10^5-10^6 cells growing axenically or on bacteria were washed 2 x with water. The cell pellet was resuspended in 50 mM KCl, 1.5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.4, at 10^4 cells per µl. NP-40 (10% v/v) and 50 mg/ml proteinase K were added to final concentrations of 0.5% and 0.05 mg/ml, respectively, incubated at 56 ˚C for 45 min, and then incubated at 95 ˚C for 10 min. Oligonucleotides were synthesized at the UF ICBR DNA Synthesis lab, and used to prime the PCR amplification of plasmid DNA or gDNA isolated from growing *Dictyostelium* cells. 2-3 µl aliquots of the above samples were used for 15-20 µl PCR reaction with following cycles: 94 °C, 3 min; [94 °C, 45 sec; 49°C, 1 min 30 sec; 72 °C 2-3 min] 30-35 cycles, 72 °C; 7 min. PCR products were analyzed on 1.2% Seakem agarose in TAE buffer. For sequencing, PCR products were separated on 1% Seakem GTG agarose gel purified with gel extraction kit (Qiagen), and sequenced using PCR primers at the ICBR core lab in University of Florida. Automated DNA sequencing was performed using the Perkin Elmer/Applied Biosystems Model 373A. The fluorescent four-dye labeled extension products were separated on Acrylamide gel and analyzed with a real-time scanning detector.

Plasmid Construction

PCR primers were designed to include a BamHI site on their 5' end to facilitate cloning into the BamH I site of pET11a vector (Novagen). PCR products, corresponding to the N-, C-terminus and N/C-hybrid of SP85, were made from p14E6Pst73#1 amplified in *E. coli* strain DH5α (Powell-Coffman and Firtel, 1994). PCR products were digested with BamH I for insertion into the BamH I site of pET11a vector. The DNA ligates were transformed into DH5αMCR competent cells. Colonies were screened by PCR (Runnebaum et al., 1991) with primers in T7 promoter and the insert. Plasmids were prepared with a Qiagen miniprep kit for sequence confirmation and transformation into BL21 (DE3) expression strain.

Protein Expression and Purification in *E. coli*

Colonies of BL21 (DE3) were inoculated in 50 ml LB medium with 100 μg /ml Ampicilin at 30 °C to OD 0.6 (600 nm) and induced for expression with 1 mM IPTG for . 2-3 h. Cells were then collected and suspended in 50 mM Tris-HCL, 2 mM EDTA, pH 8.0 (TE buffer). The cell suspension was sonicated for about 3 X 1 min pulses in an ice bath till the solution became clear . Soluble cell fractions were separated from inclusion bodies and other cell debris by centrifugation at 16 kg force for 20 min. The pellet was washed sequentially with 1 M NaCl, 0.1% Triton-X 100 in TE buffer and TE buffer only to purify inclusion bodies (Mukhopadhyay, 1997), which were then solubilized with 8 M urea, 50 mM DTT in TE buffer, at 22 °C for 1 h. The urea extract was diluted with TE buffer four fold and placed at 4 °C overnight. Subsequently, a 2 ml aliquot was removed for desalting with PD-10 column. To the rest, 5 mM reduced glutathione and 1 mM oxidized glutathione were added (Orsini and Goldernberg, 1978; Buchner and Rudolph, 1991). The new solution was placed at 4 °C 16-24 h before being applied to PD-10 column. The column was equilibrated and eluted with 20 mM Tris, pH 7.4, 5 mM DTT.

Results

Isolation and Extraction of Coats from Pre-germination and Germinated Spores

To analyze coats before they are altered by germination, spores were lysed in a French pressure cell, and the coats were purified by density gradient centrifugation exactly as for their germinated counterparts. The coat preparations were homogeneous and indistinguishable from germinated coats under phase contrast optics. Examination of sections of embedded material in the transmission electron microscope confirmed the homogeneous nature of the preparation (Fig. 2-1A), and revealed that the inner side of coats were nearly always attached to a membrane (Fig. 2-1B), probably the plasma membrane with which they are associated in the original spore. The coat seemed to stabilize the membrane because its appearance was not altered by treatment with 0.1% NP-40 (Fig. 2-1C). Despite its apparent high affinity for the coat, the membrane was often pleated (Fig. 2-1C) whether or not it was treated with NP-40, apparently to accommodate a tendency of the coat to curl after release from the spore. Cellulose was previously shown to lie in the central region of the coat deep to the outer proteinaceous layer (Hemmes et al., 1972; Orlowski and Loomis, 1979). The outer proteinaceous layer resolved into two tracks (Fig. 2-1B, arrowhead), which correlates with the pattern of immunogold labeling using mAb 83.5 against the fucose epitope of outer layer proteins (Orlowski and Loomis, 1979). The inner proteinaceous layer (Erdos and West, 1989), adjacent to the plasma membrane, was not distinct using these staining conditions. Coats isolated after germination were compared (Fig. 2-1D) and, as observed previously (Orlowski and Loomis, 1979), were free of membrane. The protein compositions of the pre-germination and post-germination coats were similar within the resolution of SDS-PAGE analysis (data not shown). This demonstrates that there were no plasma membrane proteins or other contaminating proteins present in these coat preparations at the same quantities as the major coat proteins. Thus the pre-germination coat preparations are largely free of contamination by other structures except for the plasma membrane, and the protein composition of the coat does not appear to be altered by germination. Additionally, the plasma membrane does not contribute abundant amount of proteins to the coat/membrane complex.

Treatment with 8 M urea and 5% 2-ME at 100 °C followed by centrifugation was previously shown to solubilize 85% of the protein of post-germination coats (West et al., 1996). Monosaccharide composition analysis before and after urea extraction was performed to determine whether the polysaccharides of the coat were also extracted. Except for glucose, the monosaccharide composition of the intact coat was in reasonable agreement with the content of the sum of the urea-released material and the urea-residue (Table 2-1). Comparison of the galactose content of the urea extract compared to the extracted residue showed that less than 5% of total coat galactose was released, indicating that the previously described galuran, a galactose polysaccharide (West and Erdos, 1990) was not solubilized. Similarly, most galactosamine (72%) was not extracted, consistent with the presence of this sugar in the galuran. Most mannose/xylose (94%) was retained in the extracted coat residue, whereas most glucosamine (87%) was extracted, suggesting that this amino sugar is largely associated with protein, possibly as GlcNAc. Strong acid hydrolysis yielded >50 times more glucose than did mild acid hydrolysis, consistent with earlier studies that glucose is present as cellulose (West et al., 1996). Less than 0.25% of total glucose was extracted by hot urea/2-ME, showing that cellulose also remained insoluble. The total level of cellulose as glucose was 330 fg coat^{-1}, compared to 450 fg protein coat^{-1} (West et al., 1996). About one-third of this amount of glucose was measured in the coats before extraction. The reason for this discrepancy is not known, but may have resulted from interference by other substances in the non-extracted coat.

When urea and 2-ME were removed by dialysis or gel filtration on a PD10 column, >90% protein remained soluble after centrifugation at 100,000 g. Thus coat proteins do not re-form an insoluble complex under these conditions. Reconstitution of an insoluble complex might require cellulose and/or the galuran.

Extracted Coat Proteins Reassociated with Cellulose after Renaturation

To test the potential requirement of cellulose for protein to form an insoluble complex, coat extracts were desalted by gel filtration to remove urea, and then incubated with Avicel, which consists of 5-10 µm diameter particles of crystalline and amorphous cellulose. After centrifugation, the bound and non-bound fractions were analyzed by SDS-PAGE followed by staining with Coomassie Blue.

After incubating 30 µg of desalted protein from pre-germination coats with 3 mg of Avicel, comparison of the bound and non-bound fractions showed that all major coat proteins bound partially (Fig. 2-2 a, b). Binding of the coat extract to Avicel was specific, as binding to polysaccharide particles of Sephadex G-75 or Sepharose CL-4B was negligible (Fig. 2-2 c-f). Bands at Mr positions 97,000 and 35,000, relative to Mr standards included on the same gel, bound somewhat more efficiently to Avicel than the other coat proteins when the band intensities are compared between the bound and non-bound fractions (Fig. 2-2 a, b). These proteins are shown below to be the previously described SP85 and SP35, respectively. The apparent Mr of SP85 differs from the value implied by its name (85,000) because the migration of this glycoprotein, whose predicted polypeptide mass is only 57,094 (see below), depends on the gel system used and the strain from which it is isolated (McGuire and Alexander, 1996; West et al., 1996). The abundant outer-layer coat protein SP96, whose identity was confirmed using mAb 83.5 (West and Erdos, 1990), bound least efficiently, which mimics the inefficient incorporation of SP96 during coat formation, being found mostly in the interspore matrix (West et al., 1996; Wilkinson and Hemmes, 1983). Similar results were obtained for urea extracts of germinated coats.

SP85 and SP35 Bound Cellulose after Partial Purification

To determine whether individual proteins such as SP85 and SP35 have intrinsic binding activity or whether binding is dependent upon a mixture of all proteins as suggested by a previous study (McGuire and Alexander, 1996), proteins were partially purified and assayed for Avicel binding.

Based on 2-D gel electrophoresis (West et al., 1996), DEAE-chromatography at pH 6.0 was predicted to adsorb all coat proteins except for the neutral species SP87, SP85, SP65 and SP35. As expected, bands corresponding to these proteins appeared in the flow-through fraction when the pre-germination coat extract was applied to a column equilibrated without urea. SP96, detected with mAb 83.5, eluted at 0.6-0.7 M NH_4Ac, consistent with its acidic apparent pI (West and Erdos, 1990). Elution of the other coat proteins required 6 M urea (Gonzalez-Yanes et al., 1989, West and Erdos, 1990).

The DEAE-flow through fraction was further resolved on a SP-cation exchange column equilibrated in 6 M urea at pH 5.0. The column was eluted first with a salt gradient in 6 M urea followed by an ascending pH gradient in 4 M urea. SDS-PAGE analysis showed that SP35, SP65, SP85 and SP87 eluted sequentially in partially overlapping fashion in the salt gradient. Similar protein bands were also eluted near the end of the pH gradient. Without urea in the elution gradients or at pH 6.0, these proteins eluted more broadly and were extensively degraded. Extensive degradation was also observed if the original urea extract was applied to the SP-column before passage over the DEAE-column (Data not shown).

To test binding of these proteins to Avicel, individual FPLC fractions were concentrated in 0.5 ml Microcon centrifugal ultrafiltration cartridges so that urea was diluted to 50-100 mM in the assay. Minimal binding to the cellulosic ultrafiltration membrane occurred in 6 M urea. In fractions from pre-germination coat extracts which were highly enriched in SP85, SP85 bound cellulose efficiently, as shown by the greater amount of the SP85 band in the bound fraction (Fig. 2-3, lane b) compared to the corresponding non-bound fraction (lane a). The bound:non-bound ratio was a more valid measure of binding than the absolute amount of bound material, because most coat proteins exhibited a basal level of binding to Avicel and other polysaccharide matrices which was proportionate to the total amount of the protein. The identity of the Coomassie Blue stained band as SP85 was confirmed by Western blot detection with mAb 16.1 (lanes c, d). Other fractions enriched in SP65 (lanes e, f) or SP87 (lanes g, h) showed that these proteins bound inefficiently. However, in fractions which contained both SP65 and SP85, SP65 binding to cellulose was also detected (lanes a, b), which may indicate an interaction between SP65 and SP85. SP85 isolated from post-germination coats also bound cellulose efficiently (lanes k, l), despite that in this case SP85 bound to the DEAE column and partially co-eluted with SP96. Coomassie Blue analysis of binding in this fraction showed that SP96 did not bind cellulose (lanes i, j), while the slightly more rapidly migrating SP85 band, though overlapping with SP96 in the unbound lane (lane i), was selectively enriched in the bound fraction. Another fraction containing SP96 also did not bind cellulose (lanes q, r). SP35 from fractions of pre-germination coats which were highly enriched in this protein bound efficiently to Avicel, as determined by Coomassie Blue staining (lanes m, n). Similar results were observed in fractions from post-germination coats (lanes q, r) which were enriched in SP35 co-eluting with SP85 from this extract.

Among the purified proteins examined, cellulose-binding seemed to be specific for SP85 and SP35, since it was not observed for other coat proteins. Binding seemed to be intrinsic to SP85 and SP35, since no other proteins were present at similar levels in the FPLC fractions. In addition, binding occurred whether they initially flowed through (pre-germination) or bound to (post-germination) the DEAE column. These results suggested that no other molecules are likely to be required for the cellulose-binding of SP85 or SP35 because they would be unlikely to copurify in both circumstances.

To verify that SP85, SP65 and SP35 from pre-germination coats were equivalent in charge to their previously described counterparts from post-germination coats (West and Erdos, 1990), they were examined by 2-D gel electrophoresis. The DEAE flow-through fraction was concentrated, desalted, and applied to an Avicel column. The column was eluted sequentially with water, 1 M NH_4Ac, 8 M urea, and 0.1% SDS, each containing 2-ME. SDS-PAGE analysis showed that the majority of SP85, SP65 and SP35 eluted in the urea fraction, with the remainder eluting in the flow-through and SDS fractions. Analysis of the urea-eluted fraction by 2-D O'Farrell gel electrophoresis under denaturing conditions revealed that SP85, SP35 and SP65 each migrated to the neutral end of the gel similar to their behavior in extracts of post-germination coats (West and Erdos, 1990). Thus differential binding to DEAE of SP85 and SP35 from pre- and post-germination coats could not be attributed to changes in intrinsic charge.

SP85 Is Encoded by the *psp*B Gene

Gel bands from fractions enriched in SP85 were treated with CNBr to generate internal peptides, as sequence could not be obtained by Edman degradation of the intact protein. SDS-PAGE-purified CNBr-peptides yielded four sequences (Table 2-2) belonging to the previously described prespore cell-specific *psp*B (14E6) gene (Powell-Coffman and Firtel, 1994), and four from the *psp*D (PL3 or SP87) gene (Yoder et al., 1994)). Since the SP87 protein could be detected in the SP85-enriched fraction and did not bind Avicel (Fig. 2-3g, h), these data suggested that SP85 is encoded by *psp*B.

The previously published sequence of *psp*B (Powell-Coffman and Firtel, 1994) was derived from the genomic library plasmid pG219, amplified in *E. coli*. Our sequencing of this plasmid added additional nucleotides at positions 1723 and 1769 (see Fig. 2-5 for sequence), changed the identity of nucleotides at positions 802 and 1734, and extended the sequence in the 3'-direction starting at position a in the schematic diagram in Fig. 2-4. An identical sequence was found in the cDNA library plasmid p14E6Pst73#1 represented in Fig. 2-4C, except for differences in two areas: a) the central region encoding the TXPP tetrapeptide repeats, and b) the 3'-end of the coding region (starting at arrow b in Fig. 2-4B) where there is a novel substitution starting at position 1877 (Fig. 2-5). Consistent with the otherwise identical sequences of the two DNAs. Southern blot analysis (Powell- Coffman and Firtel, 1994), physical mapping (Kuspa and Loomis, 1996), and genetic analysis (Smith et al., 1989) suggested that there is only a single *psp*B locus. To examine the origin of the different 3'-sequences, oligonucleotide primers corresponding to each 3'-sequence (P7 for p14E6Pst73#1; P8 for pG219) were combined with the primer P1 matching a shared upstream sequence (see Fig. 2-4A for primer positions), and used to amplify native gDNA from strain Ax3 in a PCR reaction. The primer pair P1/P7 amplified a product from gDNA, but P1/P8 did not, indicating the sequence in cDNA plasmid is derived from the *psp*B gene locus. However, this product was about 500 bp longer than expected, which is examined below. The divergent 3'-sequence in pG219 is probably non-contiguous *Dictyostelium* DNA. It is unlikely to represent an alternative exon, as a) no intron was seen at this position in the genomic library DNA, b) introns are short in *Dictyostelium* so the PCR reaction would be expected to bridge the hypothetical intervening sequence(s) yielding a slightly longer product, c) if a hypothetical intron were located at the point of divergence between the two sequences, primer P4 would have been unable to hybridize, as it bridges this position, and d) the new sequence in Fig. 5, but not the pG219 sequence, has been found in the Tsukuba cDNA sequencing project.

As described above, PCR amplification of gDNA using P1 and P7 yielded a product of 2.1 kb which was 0.5 kb longer than expected. A similar discrepancy was also observed using P1 and P5 (shown below in Fig. 2-6A), but not P1/P6 and P3/P4, which localized the difference to the coding region between P2 and P3 encoding the TXPP tetrapeptide repeats, where the two library sequences also diverged (Figs. 2-4A-C). Direct sequencing of the PCR-amplified gDNA between P1 and P4 revealed sequence identity with p14E6Pst73#1 except for 550 nt of additional, novel sequence starting at nt 613 (Fig. 2-5), within the tetrapeptide repeat region. This sequence included 458 nt in-frame with the previously identified ORF, and 92 nt of intron-like sequence (Figs. 2-4A, 2-5). The putative intron consisted primarily of homopolymeric stretches of A's and T's, began with a GA sequence at the second position of the codon triplet, and ended with an AT, thus conforming to the typical *Dictyostelium* intron pattern. As observed for other coat protein genes (West et al., 1996), this intron is positioned at the junction between distinct sequence motifs suggestive of separate folding domains. Conceptual translation of the additional sequence showed that it encoded a C4C-type Cysteine-repeat motif nested within 28 TXPP tetrapeptide repeats. C4C-type repeats are also present elsewhere of SP85 and other coat proteins (West et al., 1996). Inclusion of the new sequence does not cause the total known sequence to exceed the length of the mRNA as determined by Northern blotting (Powell-Coffman and Firtel, 1994). It is surmised that amplification of *psp*B library DNA in *E. coli* resulted in loss of internal sequence resulting from recombination of the highly repetitive DNA encoding the TXPP tetrapeptide repeats. The inferred recombination events deleted nts 870-1296 from *psp*B in pG219, and nts 810-843 and 954-1296 from *psp*B in p14E6Pst73#1 (Figs. 2-4A-C, 2-5), thus explaining the length discrepancies between the two plasmids in this region.

The revised sequence suggests that SP85 consists of three domains separated by 17 and 10 TXPP repeats, respectively (Fig. 2-4). The middle domain is comprised of a single EGF-like C4C-repeat. Four additional C4C-repeats occur in the C-terminus.

Polymorphism of SP85 Is Due to *psp*B Allelic Variation in the Number of TXPP Repeats

SP85 displays Mr polymorphisms in wild-type strains WS380B and WS576 relative to NC-4 and its axenic derivative Ax3 (Smith et al., 1989; Fig. 2-6B). PCR analysis of gDNA from these strains using P1 and P5 revealed a corresponding variation in the lengths of *psp*B DNA (Fig. 2-6A), verifying that SP85 is encoded by *psp*B. Sequence analysis of the PCR products revealed that *psp*B from all 3 strains diverged in the region of TXPP tetrapeptide repeats g-q (Fig. 2-4), such that 2 and 3 fewer repeats were encoded in 380B and WS576, respectively. These were not simple deletions from the Ax3 sequence, but may have been deletions from a hypothetical parent strain with at least 4 more repeats than found in Ax3. Thus this region of *psp*B, which was recombinogenic in *E. coli*, also displays heterogeneity in different wild-type strains of *Dictyostelium,* as predicted previously (Smith et al., 1989), and also as described for another *Dictyostelium* gene encoding repetitive mucin-like repeats (Gooley et al., 1992). Spontaneous recombination is infrequent, however, as no differences were seen between strain NC-4 and its derivative Ax3.

SP35 Is Encoded by the *psv*A Gene

CNBr-peptides from pre-germination SP35 gel bands and SP35 from post-germination coats yielded six amino acid sequences (Table 2-2). These sequences were each found in the 5'-half of the previously described prespore cell-specific *psv*A (EB4) gene, and defined SP35's N-terminus after signal peptide cleavage. Since psvA appears to be a single-copy gene based on Southern blot analysis (Hildebrandt et al., 1991a) and physical mapping (Kuspa and Loomis, 1996), SP35 is likely encoded by *psv*A. However, the apparent Mr of SP35 was smaller than both the predicted value for the *psv*A gene product p58, which is 53,015, and its previously reported value, 58,000 (Hilderbrand et al., 1991a). To analyze the difference, SP35 was probed with antiserum specific for the NH_2-terminal and COOH-terminal regions of p58. Only the C-antibody, generated against a peptide corresponding to the Cys-rich NH_2-terminal part of p58, recognized SP35 (Fig. 2- 3 lanes o, p). This suggest that SP35 is encoded by the 5'-part of the *psv*A coding region. This was supported by amino acid composition analysis, which can be a sensitive measure of protein identity (Shaw, 1993) and showed a close match to the predicted composition of codons 23-291 of *psv*A (Fig. 2-7). The predicted Mr, 28,834, is consistent with the measured apparent Mr of 35,000. Since p58 is absent from spores (Hildebrandt et al., 1991a), and sequences from the 3'-coding region of p58 but not SP35 have been found in the Tsukuba cDNA cloning project, p58 is probably proteolytically cleaved in the vicinity of amino acid 291 to yield SP35 during sporulation.

The p58 and SP35 products of the *psv*A gene are represented in Figs. 4G and H. SP35, which corresponds to the N-terminal half of p58, consists primarily, as previously noted (Hildebrandt et al., 1991a), of seven C4C EGF-like repeats.

The C-terminus of SP85 Bound Cellulose as Partial *psp*B Product Expressed in *E. coil.*

For further evidence that SP85 has intrinsic cellulose-binding activity, the construct shown in Fig. 2-4D, which corresponds to the *psp*B derivative represented in the cDNA library plasmid p14E6Pst73#1, was expressed in *E. coli*. This construct encoded the N-terminal domain upstream of and including 11 of the tandem TXPP repeats, and the C-terminal domain. Constructs encoding the N- and C-terminal domains separately (Figs. 4E, F) were also expressed. The terminal domain constructs were devoid of TXPP repeats as they failed to be expressed in *E. coli*. These constructs lacked the *psp*B signal peptide, and encoded a T7-epitope tag at the N-termini.

E. coli clones expressing the N/C *psp*B cDNA were lysed and separated into soluble and insoluble (inclusion body) fractions. The inclusion body fraction contained a predominant protein by SDS-PAGE/Coomassie Blue analysis with an apparent Mr of 50,000 (Fig. 2-8, lanes g-n). This was consistent with the expected Mr of 49,309, and the protein band reacted with the anti-T7 antibody. The soluble *E. coli* fraction contained lesser amounts of the Mr 50,000 protein and a smaller Mr 25,000 protein, whose level increased at the expense of the Mr 50,000 band in older extracts and during incubation in the absence of cellulose (lanes c-f). Since this smaller fragment carried the N-terminal epitope tag, it is likely to be a proteolytic derivative containing the N-terminal domain only. *E. coli*. clones transformed with the N-terminal fragment construct contained an anti-T7 reactive protein with an apparent Mr of 24,000 in both the soluble fraction and in inclusion bodies (lanes s-z), in close agreement with the predicted Mr of 24,151 for the N-terminal region of SP85. Clones expressing the C-terminal fragment contained an anti-T7 reactive protein of apparent Mr 21,000 in inclusion bodies (lanes o-r), close to the expected Mr of 21,688.

Both the soluble and urea-solubilized inclusion body fractions from the N/C-expressing strain were tested for binding to Avicel and Sephadex (Fig. 2-8), as for coat proteins in Fig. 2-2 and 2-3. The N/C-fragment from the soluble fraction bound Avicel strongly (lanes c, d), as determined by Western blotting with the anti-T7 epitope Ab. Binding was specific for cellulose, as the fragment did not bind Sephadex (lanes e, f), and was specific for the N/C-fragment, as other Coomassie Blue stained bands, as well as the accumulated truncated N-terminal SP85 fragment (N'), failed to bind Avicel (lanes a, b). The N/C-fragment recovered from the insoluble fraction also bound Avicel efficiently and selectively, but only if it had been treated with glutathione (lanes g-n). This material showed partial binding to Sephadex (lanes m, n), as if it had not fully renatured. Approximately 2.5 μmol of the renatured N/C-fragment bound per g of Avicel at saturation, which is comparable to the binding of known cellulose-binding proteins

(Goldstein and Takagi, 1993). These results confirmed that SP85 binds Avicel. The cellulose-binding activity of SP85 appeared to depend on protein folding, and did not require the middle domain containing the C4C-1-repeat and the TXPP repeats, and glycosylation.

The expressed C-terminal fragment, found only in the insoluble fraction, also bound Avicel (lanes o-r), but only when it was treated with glutathione. The expressed soluble N-terminal fragment failed to bind Avicel (lanes s-v), consistent with observations on the N'-fragment above. The N-fragment derived from the insoluble fraction showed only non-specific binding to Sephadex and Avicel (lanes w-z) even when treated with glutathione. Thus the Avicel binding of the N/C-protein could be ascribed to its C-terminal domain, and the non-specific binding of some fractions to Sephadex could be ascribed to its N-terminal domain, which may not have fully renatured.

SP85 and SP35 Are not Cellulase

Since many known cellulose-binding proteins degrade cellulose (Goldstein and Takagi, 1993; Ramalingam et al., 1992; Tomme et al., 1995; Gilkes et al., 1991) or bind to cellulases (Bayer et al., 1996; Morag et al., 1995), we asked if SP85 or SP35 possessed or associated with cellulase activity in any of the extracts. Cellulase activity was measured by a viscometric assay (Table 2-3), which measures the digestion of carboxymethyl-cellulose in solution, and a zymogram assay (Fig. 2-9), which measures the digestion of carboxymethylcellulose overlayed on an SDS-PAGE gel as revealed by negative staining with Congo-red, while proteins in the gel are positively stained.

Of the various spore-associated fractions, cellulase activity was detected only after germination, in the dialysis-renatured urea extracts of coats, and the medium in which germination occurred (Table 2-3). As found previously (Jones et al., 1979;

Roseness, 1968), prior to germination, coat extracts (Fig. 2-9B) and the interspore matrix from between spores (Table 2-3) exhibited little or no activity. Nearly all of the activity in the coat extract migrated with an apparent Mr of 75,000 and could bind to Avicel (Fig. 2-9B). This band is distinct from SP85 and SP35 and referred to as Cel1. Cel1 is similar to a previously described cellulase, CelA, which is synthesized and secreted during germination (Blume and Ennis, 1991).

Cel1 was detected after DEAE-chromatography of the urea extract of post-germination coats when the extract was pre-dialyzed, co-eluting with SP85 (Table 2-3). Since no activity was found in the absence of pre-dialysis (Table 2-3), co-elution did not seem to reflect an intrinsic property of the cellulase activity. Zymogram analysis showed that the cellulase corresponded to Cel1 (Fig. 2-9A). Thus SP85 and SP35 do not possess intrinsic cellulase activity.

Discussion

Reconstitution of an insoluble coat protein complex required cellulose. Spore coats could be isolated either from intact spores after lysis in a French pressure cell or after natural germination. When total protein was extracted using urea and reducing agent, the cellulose and galuran were retained in the residual shell (Table 2-1). After removal of urea and reducing agent by gel filtration or dialysis, the protein largely remained soluble after centrifugation at 100,000 g x 30 min. This was consistent with earlier data showing that coat proteins secreted during suspension culture, in the absence of spore differentiation, are soluble (Erdos and West, 1989). However, when incubated with insoluble particles of Avicel type cellulose under nondenaturing conditions, proteins formed an insoluble complex (Fig. 2-2a,b). Incubation of coat proteins renatured by gel filtration with a 100-fold mass excess of cellulose captured half of the protein, showing that renaturation was efficient. The galuran, which was not extracted by urea (Table 2-1), was thus not required for cellulose-binding. All major protein species were bound (Fig. 2-2a), suggesting that reconstitution was similar to the original process of coat formation. Proteins did not bind to Sephadex or Sepharose (Fig. 2-2c-f) demonstrating their specificity to cellulose consistent with the polysaccharide composition of the coat.

During the course of these experiments, it was observed that proteins renatured by dialysis instead of gel filtration bound cellulose poorly although more efficiently than to Sephadex or Sepharose. SP35 was greatly depleted after dialysis and could be recovered from the cellulosic dialysis membrane by washing with SDS and reducing agent, and this eluate was greatly enriched in SP35 and SP85. Indeed, these two proteins were also the most enriched in the cellulose-binding fraction of coat extracts renatured by gel filtration (Fig. 2-2a, b). This suggested that binding of most coat proteins to cellulose might be indirect via SP35 and SP85, but this model remains to be tested. In the present study, it was necessary to avoid dialysis and ultrafiltration through cellulosic membranes, except in the presence of 4-8 M urea, to ensure protein recovery.

In another study, coat protein precursors isolated from prespore cells exhibited poor binding to cellulose (McGuire and Alexander, 1996). A possible explanation for the divergent results is that cellulose-binding may be developmentally regulated, e.g., p58 was not yet replaced by SP35. Alternatively, low binding may have been due to inappropriate disulfide bond formation during prespore cell lysis (West et al. 1996), or the presence of non-ionic detergents, which might interfere with hydrophobic interactions in cellulose-binding (Gilbert et al., 1990; Renikainen et al., 1992; Sakka et al., 1996).

SP85 is encoded by *psp*B. The Mr 85,000 coat protein which binds cellulose is equivalent to the previously identified SP85 coat protein (West et al., 1996), also known as PsB, based on similar apparent Mr, Mr polymorphisms in different wild-type strain isolates, apparent pI, and reactivity with mAbs 16.1 and MUD102. SP85 is encoded by *psp*B, based on a perfect match of internal SP85 peptide sequences with translated *psp*B sequences, and correlation of SP85 Mr polymorphisms with sequence variations in *psp*B, in different strains. *psp*B was previously concluded not to encode a coat protein based on the absence of differences in coats from a putative *psp*B$^-$ strain (Powell-Coffman and Firtel, 1994; West et al., 1996). Based on the new information in this report showing that a region comprising 550 nts was missing from the plasmid previously used to target the *psp*B locus, it is not surprising that the previous gene targeting strategy (Powell-Coffman and Firtel, 1994) was not successful.

The DNA encoding the TXPP tetrapeptide repeats appears to be recombinogenic in *E. coli* as both previously reported library copies of *psp*B were missing the majority of the TXPP tetrapeptide repeats, including the embedded middle domain C4C repeat and intron (for the genomic library copy) (Fig. 2-4B, C). This region also appears to be recombinogenic in *Dictyostelium*, as it is shorter by 2 or 3 TXPP repeats in wild-type strains WS380B or WS576, respectively. This was previously predicted from a genetic analysis of the *psp*B locus (Smith et al., 1989), which is shown here to be equivalent to the molecularly defined *psp*B (14E6) locus (Powell-Coffman and Firtel, 1994), having coincidentally been given the same name. Similar polymorphisms have been observed for another *Dictyostelium* protein containing tandem PTVT-tetrapeptide repeats, SP29 or PsA (Gooley et al., 1992), and for vertebrate mucin genes (Verma and Davidson, 1994).

Based on the revised sequence of *psp*B, SP85 is proposed to contain three major domains separated by 17 and 11 tandem TXPP tetrapeptide repeats, respectively (Fig. 3A). The TXPP repeat clusters may serve as interdomain spacers as suggested for similar sequences in other extracellular proteins (Gilkes et al., 1991; Gooley et al., 1992; Ramalingam et al., 1992). The N-terminal domain bears no obvious resemblance to other database sequences. The middle domain consists of a single C4C-type repeat with a characteristic spacing of Cys-residues and predicted β-turns, and resembles the N-terminal subdomain of the EGF-motif (Hildebrandt et al., 1991a; West et al., 1996). C4C-repeats are prevalent in all other known coat proteins (West et al., 1996) and is also found in a stalk matrix protein, *sta*B (Robinson and Willians, 1996). The cellulose-binding C-terminal domain contains 4 additional C4C-repeats, two of which are separated by a short basic proline-rich sequence which frequently separates C4C-repeats in other coat proteins (West et al., 1996). The C-terminal 1/3 of the C-terminal domain sequence is unique. The *mod*B glycosylation modification may be applied to the TXPP-repeat region as similar repeats are modified in this way in another prespore cell protein, SP29 (Gooley et al., 1992; Zachara et al., 1996) and cellulose-binding proteins in other systems (Gerwig et al., 1993).

SP35 is encoded by *psv*A. SP35 was suggested to be encoded by the single-copy (Hildbrandt et al., 1991b; Kuspa and Loomis, 1996) *psv*A locus based on the absolute sequence match between internal peptides of SP35 with the conceptual translation product of *psv*A. However, the open reading frame of *psv*A predicts a protein product of Mr 53,015 after signal peptide cleavage, and an Mr 58,000 protein, p58, has been detected in prespore cells by antibodies raised against polypeptides corresponding to the N-terminal and the C-terminal halves (Hildbrandt et al., 1991a). SP35 corresponds to the 5'-half of the coding region from codons 23 to approximately 291, based on its apparent Mr of 35,000, its amino acid composition (Fig. 2-7), reactivity with an antiserum raised against the N-terminal half (Fig. 2-3o, p) but not one against the C-terminal half, and the positions of the amino acid sequences obtained. *psv*A was previously concluded to not encode a coat protein based on the observation that p58 could not be detected after sporulation using the antibody against the C-terminal half (Hildbrandt et al., 1991a). The simplest explanation is that, as prespore cells differentiate into spores, p58 is processed into SP35 which becomes a major component of the spore coat. Based on the anti-H antibodies used, this would not have been detected in the previous study. A strain in which *psv*A is thought to be disrupted exhibited no obvious phenotype (Hildbrandt et al., 1991b) but, since an Mr 31,000 *psv*A-encoded protein similar to SP35 was still produced, an SP35-like protein was probably still produced by this strain.

SP85 and SP35 bound cellulose in the absence of other protein interactions. SP85 and SP35 each exhibited efficient cellulose-binding activities after partial purification from pre-germination coats (Fig. 2-3a-h, m-p). The renatured activities exhibited sharp specificity with respect to polysaccharide type. Binding was inhibited by urea, but not by 5 mM EDTA. Since the FPLC fractions most enriched for SP35 or SP85 did not contain other Avicel-binding proteins with similar abundance on Coomassie-Blue stained SDS-PAGE gels, it is likely that these proteins bind directly to Avicel. Cellulose-binding activity was also robust after purification from post-germination coats (Fig. 2-3i-l, q-r), despite their binding to, rather than flowing through, the DEAE-column. This also suggested that cellulose-binding was not due to a co-purifying protein. A polysaccharide component is unlikely to mediate binding, as none seemed to be extracted from the coat by urea (Table 2-1). Avicel-binding was not a property of all proteins, as SP96 (Fig. 2-3i, j, q, r), SP65 (Fig. 2-3e, f), SP87 (Fig. 2-3g, h), and the entire dialyzed coat extract (data not shown) did not bind. Non-binding does not seem to result from failure of renaturation, because these proteins were able to bind after gel filtration of the original coat extract (Fig. 2-2a, b).

Since SP85 was not as completely purified from other proteins as SP35, SP85 domains were expressed from partial *psp*B cDNAs in *E. coli*. The N/C construct containing the N- and C-terminal domains bound Avicel efficiently and specifically (Fig. 2-8c-n), verifying that other coat components were not required. Binding activity could be ascribed to the C-terminal domain (Fig. 2-8o-r). The C-terminal domain contains 4 tandem C4C-repeats with distinct sequences. The Cys-spacing of the C4C repeat is found in the fungal type I cellulose-binding domain as (Gilkes et al., 1991), and the Cys-spacing of the fungal type I motif is present across two consecutive C4C repeats of SP85 consisting a CX6C10X5X9C sequence. These Cys-rich motifs may also be important for binding of SP85 to cellulose, as Avicel-binding of the recombinant C-terminal domain required renaturation in the presence of glutathione.

The cellulose-binding activity of coat proteins prepared from prespore vesicles appeared to be relatively weak, but was reportedly specific for cellulose relative to other polysaccharide particles (McGuire and Alexander, 1996). This binding activity of total coat protein was found to be reduced even further in extracts from mutant strains which were lacking the coat proteins SP96 or SP70, and binding of SP85 was not observed. The results were interpreted to mean that a complete pre-coat complex is required for cellulose-binding activity. In contrast, proteins are incorporated normally into mutant coats *in vivo* (West et al., 1996), and we have observed that proteins extracted from mutant coats (strain TL56) exhibit efficient Avicel-binding activity *in vitro*. While the reason for this discrepancy is not clear, the low level of cellulose-binding exhibited by the proteins isolated from prespore cells may not be directly comparable to the high levels of binding when isolated from mature coats.

SP85 and SP35 interact with other coat proteins. Extensive interactions among all coat proteins except for SP75 have been previously described based on co-immunoprecipitation studies performed on extracts of prespore cells (Devine et al., 1983; Watson et al., 1993, 1994), which contain all of the coat protein precursors and the galuran compartmentalized together in the prespore vesicle (West and Erdos, 1990). These results provide an explanation for how binding of SP85 and/or SP35 to cellulose might result in the binding of all other coat proteins (Fig. 2-2a, b), although these interactions have not been shown to occur *in vivo* (West et al., 1996) and remain to be demonstrated to exist at the time of sporulation.

The possibility that coat protein interactions are regulated is suggested by several observations. Binding of SP85 and SP35 to the DEAE-column after but not before germination is apparently not due to a change in these proteins, as their intrinsic apparent isoelectric points and Mrs do not vary. Partial co-elution from the DEAE-column with SP96 and Cell (Fig. 2-9, Table 2-3) suggests that interaction of SP85 with other proteins may be developmentally regulated. A separate type of regulation is suggested by the apparent processing of p58 into SP35 before the coat is assembled.

Table 2-1: Monosaccharide composition analyses of post-germination coat extracts*

Sugar type	pmol sugar 10^6 coats^{-1} (fg coat^{-1})		
	Before urea extraction	After urea extraction	
		coat remnants	released material
D-glucose	630	1800 (330)	1.6
D-galactose	25 (4.5)	30	1.2
D-galactosamine§	14 (3.1)	13	5.0
D-glucosamine§	59 (13)	7.6	53
D-mannose/xylose¶	13 (2.3)	15	0.95

* Samples were degraded in 4 M TFA, or 100% TFA followed by dilution in H_2O (D-glucose only), and aliquots were chromatographed on a Dionex PA-1 column an quantitated with a pulsed amperometric detector.

§ Conditions of hydrolysis lead to de-N-acetylation of amino sugars.

¶ Mannose and xylose were not differentiated in the analysis.

Table 2-2: NH_2-terminal and internal sequences of coat protein fractions*

Protein band	Treatment	NH_2-terminal sequence found	Matching DNA sequence (Gene/Acc.#)
SP85/ SP87	CNBr	(M_{158})‡PLQLGYGANGKNGDN GISV (2)§	*psp*B (14E6)/ S72639
		(W_{178})LIYGYTIVDI	
		(Q_{307})VFCVPDCP	
	CNBr	(A_{105})KIKECA	*psp*D (PL3)/ U25144
		(M_{202})VNDVATCLASTTGGXGLPG	
		(D_{274})XXXDVDCPDGFXXEXKD	
		(D_{314})PCKDVTCPDGFHCECKDGKT	
SP35	CNBr	(D_{22})SVNFGRG	*psv*A (EB4)/ M11028
		(N_{25})FGRGGGWGSGNQGVLCGTH YCPPGSTCESKH (2)	
		(M_{166})VNGYLQCLFSNGTAPFACGL QTCVPPQL (2)	
	none	(D_{22})SVNFGR	

* Protein bands from SDS-PAGE gels were either electroblotted (Treatment = none), or treated with CNBr in 70% formic acid and the peptides purified again by SDS-PAGE followed by electroblotting. Genes corresponding to the amino acid sequences found are listed in the right-hand column.

‡ Residue in parentheses is deduced from the gene sequence; its location is relative to the start Met.

§ Sequence obtained twice, from different SDS-PAGE bands.

Table 2-3: Cellulase activity during germination and in extracts of post-germination coats

Preparation	Amount	Drain time (sec)*
H_2O		116
Interspore matrix	13 µg protein	116
Germination supernatant	6 µg protein	102
Dialyzed urea extract of coat	6 µg protein	79
	30 µg protein	45
	60 µg protein	49
DEAE-fractions from dialyzed urea extract‡		
-2	100 µl	116
-1	100 µl	101
0	100 µl	60
+1	100 µl	61
+2	100 µl	78
DEAE-fractions from non-dialyzed urea extract‡		
-1	100 µl	118
0	100 µl	117
+1	100 µl	115
+2	100 µl	115
+3	100 µl	115
+4	100 µl	114
T. reesei cellulase	10 µg	59
	20 µg	46

* Cellulase activity was inferred from the reduction in viscosity of a solution of carboxymethylcellulose, which was measured as the drain time from a calibrated column. Shorter drain times correlate with decreased viscosity and higher cellulase activity.

‡ The fractions are numbered to facilitate comparison between different DEAE-FPLC trials. '0' corresponds to the fraction most enriched in SP85. Neighboring fractions are numbered relative to this position.

Figure 2-1. EM analysis of spore coats. Spores were either lysed by passage through a French pressure cell (A-C), or germinated (D). Coats were purified by density gradient centrifugation on renografin, embedded in Epon, and prepared for transmission electron microscopy. (A) Panoramic view of coats purified from lysed spores. (B) View of a single coat, showing the electron dense outer layer and the associated plasma membrane (arrowhead). (C) View of a coat preparation which was treated with 0.1% NP-40. Note that the membrane is retained and tends to fold into pleats (arrowhead) as the coat curls. (D) A coat from germinated spores. The outer protein layer is most electron dense and the cellulose, which is not visible here, is layered on its concave surface (West and Erdos, 1990).

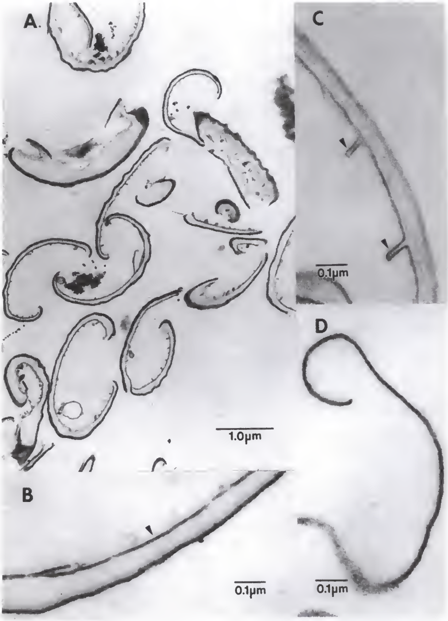

A.
1.0μm
B
0.1μm
C
0.1μm
D
0.1μm

Figure 2-2. Cellulose binding of a urea extract from pre-germination spore coats. Extracted protein was separated from urea by gel filtration, and 30 µg total protein was incubated with 3 mg of Avicel (Avi), lanes a, b; Sephadex G-75 (Sdex), lanes c, d; or Sepharose-4B (Sose), lanes e, f. Nonbound (NB) and bound (B) fractions were separated by centrifugation and then run on the same SDS-PAGE gel followed by Coomassie Blue staining. Positions of key proteins are indicated.

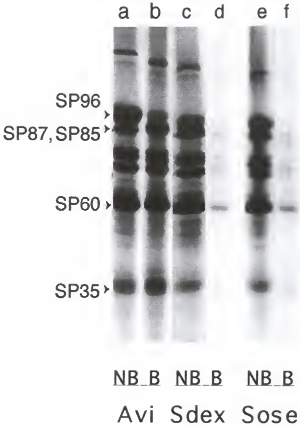

a
b
c
d
e
f
SP96
SP87, SP85
SP60
SP35
NB B
NB B
NB B
Avi
Sdex
Sose

Figure 2-3. Cellulose binding of selected FPLC fractions from spore coats. Urea extracts were fractionated by FPLC as indicated and individual fractions were tested for binding to Avicel as in Fig. 2-2. Lanes a-h: The pre-germination urea extract analyzed in Fig. 2-2 was subjected to DEAE-FPLC and the flow-through was applied to an SP-column and eluted with salt and then with increasing pH. Avicel-binding of selected fractions enriched in SP85 (a-d), SP65 (e, f) and SP87 (g, h) is shown, with detection by Coomassie Blue to show total protein in the fraction or Western blotting to confirm protein identity as indicated. Lanes i-l: A post-germination coat urea extract was dialyzed and subjected to DEAE-FPLC. SP35 is missing because it bound to the dialysis membrane. Avicel-binding of a fraction containing SP96 and SP85 (they copurified when from post-germination coats) is shown both by Coomassie Blue (i, j) and Western blotting for SP85 (k, l). Lanes m-p: Avicel-binding of a fraction from the experiment shown in lanes a-h which was enriched in SP35. Lanes q, r: Avicel-binding of a fraction enriched in SP35 and SP96, from a urea-extract of post-germination coats applied directly to a DEAE-column. Positions of key proteins are indicated.

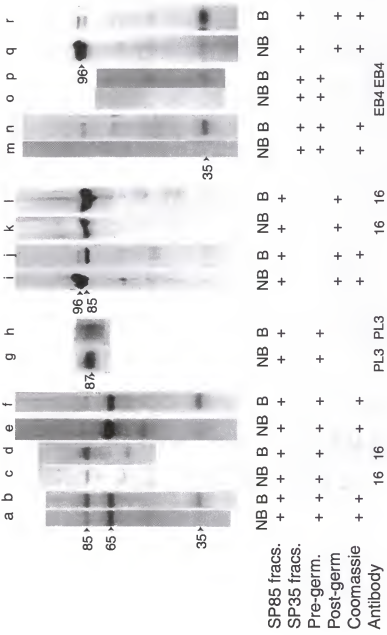

a b c d e f g h i j k l m n o p q r
85
65
35
87
96
85
35
96
NB B NB B NB B NB B NB B NB B NB B NB B NB B
SP85 fracs.
SP35 fracs.
Pre-germ.
Post-germ
Coomassie
Antibody
16 16
PL3 PL3
16 16
EB4 EB4

Figure 2-4. Organization of the *psp*B and *psv*A genes, their derived constructs, and inferred protein products. (A) This diagram is a composite of sequence derived by PCR amplification of *psp*B from strain Ax3 gDNA between primers P1 and P7, and cDNA sequence from part C below. (B) Schematic diagram of our sequence determination of *psp*B in pG219, derived from a genomic library (Powell-Coffman and Firtel, 1994) (C) Schematic diagram of our sequence determination of *psp*B in p14E6Pst73#1, derived from a cDNA library (Powell-Coffman and Firtel, 1994) (D) Construct of combined N- and C-terminal *psp*B domains expressed in *E. coli.* (E) Construct of N-terminal *psp*B domain expressed in *E. coli.* (F) Construct of C-terminal *psp*B domain expressed in *E. coli.* (G) Comparative diagram of the p58 protein product of *psv*A (Hildebrandt et al., 1991a, 1991 b). (H) Diagram of the SP35 protein product of *psv*A (see text). Tall blocks, C4C-type Cys-repeats, which are numbered in order; mid-height blocks, tandem TXPP-repeats, which are lettered in order; short blocks, other domains; lines, untranslated sequences; ss, signal sequence; solid fill, position of basic proline (BP) motif sequence; a, denotes position of previously deduced stop codon (Powell-Coffman and Firtel, 1994); b, denotes position of sequence divergence between pG219 and p14E6Pst73#1; arrows, oligonucleotide primers, which are underlined in Fig. 2-5 except for P8 in panel B, which has the sequence 5'-GTTAAGAGCATCTTCCAAAACCCTA.

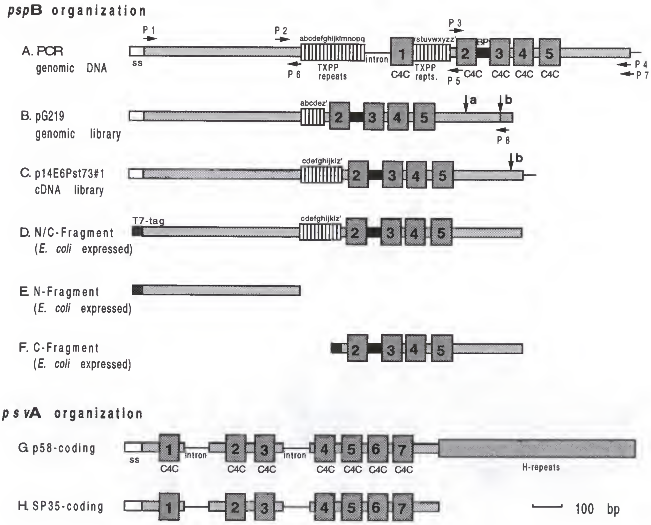

pspB organization
A. PCR genomic DNA
P 1
P 2
P 6
abcdefghijklmnopq
TXPP repeats
intron
1
C4C
rstuvwxyzz'
TXPP repts.
P 3
P 5
2
BP
3
4
5
C4C C4C C4C C4C
P 4
P 7
ss
B. pG219 genomic library
abcdez'
a
b
P 8
C. p14E6Pst73#1 cDNA library
cdefghijklz'
D. N/C-Fragment (E. coli expressed)
T7-tag
E. N-Fragment (E. coli expressed)
F. C-Fragment (E. coli expressed)
psvA organization
G. p58-coding
ss
intron
C4C C4C C4C C4C C4C C4C C4C
H-repeats
H. SP35-coding
1 2 3 4 5 6 7
100 bp

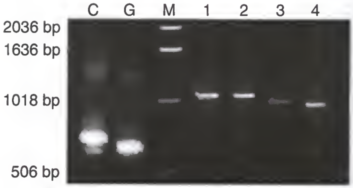
Figure 2-5. Revised sequence and deduced translation of the *psp*B coding region from strain Ax3. The sequence corresponds to the PCR-derived map shown in Fig. 2-4A, and has Genebank accession number AF066071. Partial *psp*B sequences (corresponding to nt 777-1308) from strains WS576 and WS380B (see text) have accession numbers AF066072 and AF066073, respectively. The sequence between primers P1 and P4 is derived from PCR amplification of cellular DNA (see text). The sequence upstream of primer 1 and downstream of primer 4, and the primer sequences themselves, are derived from p14E6Pst73#1. Nucleotide numbering at the 5'-end is as in ref. The sequences of oligonucleotide primers used are underlined. Amino acid sequences shown in Table 2-2 are in bold. The vertical arrow marks the position of sequence divergence between p14E6Pst73#1 and pG219 (see text).

```
                                                             P1→
222  ATGAGACTATTAAGTGTTCTTTTAATAGGATTCCTCTGTTTAGCAGGTACCTATGCACAA  281
1    M  R  L  L  S  V  L  L  I  G  F  L  C  L  A  G  T  Y  A  Q   20

282  AAATACCAATTGTCACCGGCTTATAATGATCCATATTTAACCGATGATAAAACAGGAACA  341
     K  Y  Q  L  S  P  A  Y  N  D  P  Y  L  T  D  D  K  T  G  T   40

342  CATGATTTCTGGGTTCAAAATGCGTCTTTACCAGTTTTCTATGGTTTCCATGATTGGAAT  401
41   H  D  F  W  V  Q  N  A  S  L  P  V  F  Y  G  F  H  D  W  N   60

402  TTCCAAGATAATTCTGGTATAATGGAAATTAATGGTGATGAAATGCATATCACAGGTAAA  461
41   F  Q  D  N  S  G  I  M  E  I  N  G  D  E  M  H  I  T  G  K   80

462  ATTTATCCAACTGTTAATATGGGTGATTGCCACAGATACAATGTTGATTTAGTATTCAAG  521
81   I  Y  P  T  V  N  M  G  D  C  H  R  Y  N  V  D  L  V  F  K   100

522  AAAGATAAATCTGGTAATGTTATGCCAAAGAAAGAGCTAAGAGAAAGTGCATACGTTCCA  581
101  K  D  K  S  G  N  V  M  P  K  K  E  L  R  E  S  A  Y  V  P   120

582  CATGGACCAATTGACCCAGCCACTTGGAAATATTATACTTTTGTTCAAGGTAAATGGACT  641
121  H  G  P  I  D  P  A  T  W  K  Y  Y  T  F  V  Q  G  K  W  T   140

642  GGTTTTGGTTGTGACCCTCAAAATGTCGTCTTTTCCGGTGCTGAAGGTGGTATGCCATTA  701
141  G  F  G  C  D  P  Q  N  V  V  F  S  G  A  E  G  G  M  P  L   160
                                                     P2→
702  CAACTCGGTTATGGTGCAAATGGTAAAAATGGTGATAATGGTATCTCTGTTTGGTTAATT  761
161  Q  L  G  Y  G  A  N  G  K  N  G  D  N  G  I  S  V  W  L  I   180
                                                      ←P6
762  TATGGTTACACTATTGTTGATATCAATTGTAACATTCGTCCAATCATTACACAATCACCA  821
181  Y  G  Y  T  I  V  D  I  N  C  N  I  R  P  I  I  T  Q  S  P   200

822  ACTCAACCACCAACACAACCACCAACTTATCCACCAACACAACCACCAACACAACCACCA  881
201  T  Q  P  P  T  Q  P  P  T  Y  P  P  T  Q  P  P  T  Q  P  P   220

882  ACACAACCACCAACTTATCCACCAACTTATCCACCAACTTATCCACCAACTTATCCACCA  941
221  T  Q  P  P  T  Y  P  P  T  Y  P  P  T  Y  P  P  T  Y  P  P   240

942  ACTTATCCACCAACTCATCCACCAAGTTATCCACCAACTTATCCACCAACACAACCACCA  1001
241  T  Y  P  P  T  H  P  P  S  Y  P  P  T  Y  P  P  T  Q  P  P   260

1002 ACTCAACCACCAGTACAAGgtaaagtattattttaaattatataaaatccataaaattaa  1061
261  T  Q  P  P  V  Q  D                                          267

1062 aaactcatttgttaatttctttttttttttttttttaaaaaaataataagATTGTTCAAC  1121
268                                                   C  S  T   270

1122 TTTAGAATGCCCAGAAGGTTTCCATTGTGAAATTGTAAATAATCGTAGAACATGTGTTTG  1181
271   L  E  C  P  E  G  F  H  C  E  I  V  N  N  R  R  T  C  V  C  290

1182 TGATACTACAGTTCCACCAACTCATCCACCAACTCAATCACCAACTTATCCACCAACTCA  1241
291   D  T  T  V  P  P  T  H  P  P  T  Q  S  P  T  Y  P  P  T  Q  310

1242 ACCACCAACTCAACCACCAACTTATCCACCAACTTATCCACCAACTTATCCACCAACACA  1301
311   P  P  T  Q  P  P  T  Y  P  P  T  Y  P  P  T  Y  P  P  T  Q  330
            P3→                     ←P5
1302 ACCACCAAGAGCATCATGCGATAATGTTAGATGTCCAAGAGGTTACCATTGTGAATGTAA  1361
331   P  P  R  A  S  C  D  N  V  R  C  P  R  G  Y  H  C  E  C  N  350

1362 TCATTGGGAAAATGTTGCTAGATGTGTAAGAAACGAAGAACCAACTCATAGACCAAAACC  1421
351   H  W  E  N  V  A  R  C  V  R  N  E  E  P  T  H  R  P  K  P  370

1422 ACCACACCATGGTTGTCGTCCAGATAGTTGCGCTAGAGGTGAGAAATGTATCTGTGTACG  1481
371   P  H  H  G  C  R  P  D  S  C  A  R  G  E  K  C  I  C  V  R  390
```

```
1482 TGGTAAGATCTATTGTATTCCAAAGACCACCTGTGATCAAGTACGTTGTCCAAGAAAACA 1541
391   G  K  I  Y  C  I  P  K  T  T  C  D  Q  V  R  C  P  R  K  H  410

1542 CCATTGTGAATGCAATAGAAAAGGTCAAGTTTTCTGTGTTCCAGATTGTCCAAAATTAAC 1601
411   H  C  E  C  N  R  K  G  Q  V  F  C  V  P  D  C  P  K  L  T  430

1602 TTGTAAACAAGTTGGTTGCCCAGAAAACCATGAATGCGTTTCAAGACGTGGTGAACTCCA 1661
431   C  K  Q  V  G  C  P  E  N  H  E  C  V  S  R  R  G  E  L  H  450

1662 TTGTGTATACGTTAGACCACCAACAGGTAGATGGGGTGATGATCTCTCAGATTTAGGTTA 1721
451   C  V  Y  V  R  P  P  T  G  R  W  G  D  D  L  S  D  L  G  Y  470

1722 CAATCAAGCAAGTGTTGAAGCTGCAATTGATGCTTATGAAGCCCAAAGGGGTCAAAGACC 1781
471   N  Q  A  S  V  E  A  A  I  D  A  Y  E  A  Q  R  G  Q  R  P  490

1782 ACATAATAATATTCCATCAAACAATTTACATCAAGAAAACAATGATAATTTGGGTGATGG 1841
491   H  N  N  I  P  S  N  N  L  H  Q  E  N  N  D  N  L  G  D  G  510
                                                            ↓
1842 TTTTTATGATGGTGTTGAAATTGGTTTCGCTGACGGAGGTTTCGACGTAAACGATCTCAA 1901
511   F  Y  D  G  V  E  I  G  F  A  D  G  G  F  D  V  N  D  L  N  530
       ←P4     ←P7
1902 TGGTTTTTAAAAAAAAAAAAAAAAAATAATTATTAAATTTTTA                  1944
531   G  F  Stop                                                  532
```

Figure 2-6. Strain-specific length polymorphisms in *psp*B and SP85. (A) DNA from vegetative (growing) cells was amplified using primers P1 and P5, and compared on a 1.2 % agarose gel by EtBr. staining. Templates: C, *psp*B cDNA plasmid p14E6Pst73#1; G, *psp*B genomic DNA plasmid pG219; lane 1, strain Ax3 (derived from NC-4); lane 2, strain NC-4; lane 3, strain WS380B; lane 4, strain WS576. Lane M contains an Mr ladder, whose lengths are given at the left. (B) 10^6 spores from each strain were extracted and resolved by SDS-PAGE under reducing conditions, Western blotted, and immunoprobed with mAb 16.2. The apparent Mr of SP85 from strain Ax3 is given on the left.

(A) PCR products from P1 and P5



(B) Western blot with mAb 16.2

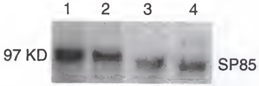


Figure 2-7. Amino acid analysis of SP35. The Avicel-bound subfraction of salt gradient-eluted SP-fractions containing SP35 were pooled and resolved by SDS-PAGE (see Fig. 2-3n), and electroblotted onto a PVDF membrane. The Coomassie Blue-stained SP35 band was cut from the PVDF electroblot and acid hydrolyzed. PTC-derivatives were prepared and analyzed by HPLC to quantitate the amino acids released. Filled circles: values which could be reliably quantitated given the amount of material available are reported. Open circles: theoretical values for codons 23-291 of the *psv*A gene. Open squares: theoretical values for codons 23-514 of *psv*A.

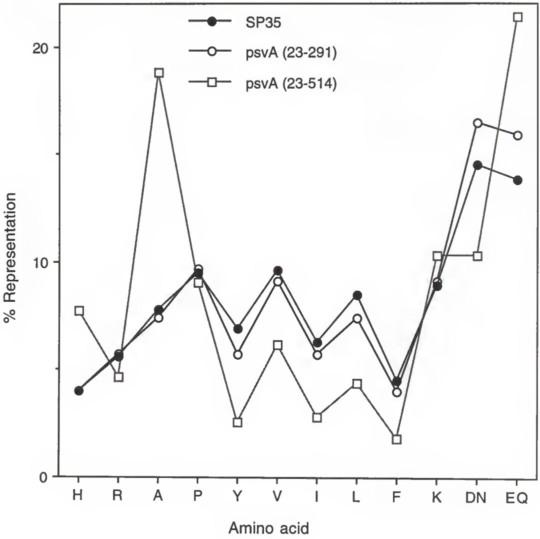
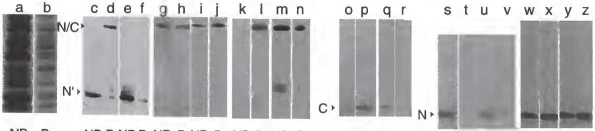
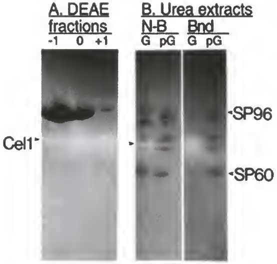

SP35
psvA (23-291)
psvA (23-514)
% Representation
0
10
20
H
R
A
P
Y
V
I
L
F
K
DN
EQ
Amino acid

Figure 2-8. Binding of recombinant SP85 polypeptides to Avicel and Sephadex. Binding of N/C-construct (Fig. 2-4D), and the separate N- (Fig. 2-4E) and C-terminal (Fig. 2-4F) domains, to Avicel or Sephadex was assayed as in Fig. 2-2, with detection either by Coomassie Blue (CB) for total protein or anti-T7 antibody for the epitope tag. Extracts consisted either of the soluble, cytoplasmic fraction, or the insoluble fraction after solubilization in urea and renaturation in the presence or absence of glutathione as indicated. Lanes a-n: Binding of extracts expressing the N/C hybrid (H) are shown; in some cases, especially when Avicel was absent, a truncated N-terminal (N') fragment was also detected. Lanes o-r: Binding of extracts expressing the C-terminal fragment. Lanes s-z: Binding of extracts expressing the N-terminal fragment.



	NB	B	NB	B	NB	B	NB	B	NB	B	NB	B	NB	B	NB	B	NB	B	NB	B	NB	B	NB	B	NB	B
N/C-domain	+	+	+	+	+	+	+	+	+	+	+	+	+	+												
C-domain															+	+	+	+								
N-domain																			+	+	+	+	+	+	+	+
Avicel	+	+	+	+			+	+			+	+			+	+			+	+			+	+		
Sephadex					+	+			+	+			+	+			+	+			+	+			+	+
CB	+	+																								
Western			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Soluble	+	+	+	+	+	+													+	+	+	+				
Insoluble							+	+	+	+	+	+	+	+	+	+	+	+					+	+	+	+
Glutathione											+	+	+	+	+	+	+	+					+	+	+	+

Figure 2-9. Zymogram analysis of cellulase activity in FPLC fractions and coat extracts. (A) DEAE-FPLC fractions of a dialyzed urea extract from post-germination coats were subjected to SDS-PAGE and incubated in 1 % carboxymethylcellulose for 2 hr. After staining with Congo red and destaining, cellulase activity and protein were visualized as yellow (light in the reproduction) and red (dark in the reproduction) bands, respectively. Fractions 0 and +1 are most enriched in SP85, and -1 is most enriched in SP96. The fractions correspond to the similarly numbered fractions analyzed in Table 2-3. (B) Dialyzed urea extracts from post-germination spores (G) and gel-filtered urea extracts from pre-germination spores (pG) were separated into Avicel-bound (Bnd) and non-bound (NB) fractions, and analyzed as in panel A. The positions of the primary cellulase detected, Cel1, and the coat proteins SP96 and SP60 are indicated.


A. DEAE fractions
-1
0
+1
B. Urea extracts
N-B
Bnd
G
pG
G
pG
Cel1
SP96
SP60

CHAPTER 3
A β-GLUCOSIDASE FROM INTERSPORE MATRIX OF *DICTYOSTELIUM* BINDS TO CELLULOSE *IN VITRO*

Introduction

During spore differentiation, numerous proteins and a galuran polysaccharide are exocytosed into the extracellular space. As cellulose fibrils emerge from the cell surface, the coat protein precursors appear to coalesce with the cellulose to form the coat. Proteins that fail to incorporate into the coat accumulate in the interspore matrix. Association of the proteins with the cellulose fibrils may be mediated by two cellulose-binding proteins, SP85 and SP35, as discussed in Chapter 2. However, a subpopulation of coat proteins including the cellulose-binding protein, SP85, were also present in the interspore matrix. Thus the interspore matrix may provide a source of cellulose-binding protein which failed to enter the coat and would not require denaturation and renaturation to study.

Application of the Avicel binding assay to native proteins from the interspore matrix revealed an abundant cellulose-binding protein distinct from the coat proteins SP85 and SP35. Internal peptide sequencing, amino acid composition analysis and reactivity with mAbs showed that it is equivalent to the *glu*A gene product β-glucosidase-2. This cellulose-binding activity is novel for a β-glucosidase, and was not predicted by comparison with the sequences of other known cellulose-binding proteins.

Materials and Methods

For the Avicel column assay, hydrated Avicel (Type PH-101, FMC Corporation, Philadelphia, PA) was de-fined, loaded into columns (1 ml bed volume), and equilibrated in 50 mM NH4Ac, pH 6.8. The interspore matrix from either strain X22 or Ax3 was adjusted with 1 M NH4Ac to 50 mM before being applied to the Avicel column, and was allowed to incubate in the column for 1-2 h at 22° C. Proteins were eluted from the column under gravity flow sequentially with 5 ml of 50 mM NH4Ac, pH 6.8, H_2O, 8 M urea, 50 mM NH4Ac, pH 6.8, and 1% SDS, 50 mM NH4Ac, pH 6.8 in the presence of 5% 2-ME in each of the above solutions. 1-D electrophoresis, Western blotting, amino acid composition analysis and internal peptide sequencing were performed as in Chapter 2. 2-D electrophoresis was performed as described by West (West et al., 1996).

Results

To screen for interspore matrix proteins which bound Avicel, the water soluble fraction from strain Ax3 sori (a collection of spores) was passed over a small column, which was then washed with 50 mM NH4Ac and eluted sequentially with H_2O, 1 M NH4Ac and 1% SDS with the presence of 5% 2-ME in each of the solutions. Column fractions were analyzed by SDS-PAGE followed by staining with Coomassie Blue (Fig. 3-1A). The majority of proteins did not adsorb to the column. A single predominant protein with an Mr value of 95,000 (CB95) eluted uniquely with H_2O. Along with the 95,000 band, a large number of low Mr protein species were eluted with 1% SDS/2-ME.

The protein composition of the interspore matrix varies greatly between wild type and axenic strains. To determine if the H_2O-eluted CB95 was unique to strain Ax3, the same experiment was performed on an interspore matrix preparation from wild type strain X22. As seen for strain Ax3, a predominant protein with an apparent Mr value of 95,000 eluted with H_2O (Fig. 3-1B). Proteins subsequently eluted with 1 M NH_4Ac and SDS were very different between the two strains.

The elution of CB95 by H_2O suggested that it might bind cellulose directly as for other known cellulose binding proteins (Gilbert et al., 1990; Ramalingam et al., 1992; Goldstein and Takagi, 1993). However, binding may have been indirect via other proteins which were eluted afterwards. This was tested by reapplying the H_2O-eluted fraction from Fig 3-1B to another Avicel column in the presence of 50 mM NH_4Ac, pH 7.4 /2-ME (Fig. 3-2). Only the minor lower Mr value proteins appeared in the flow-through fraction. All of the CB95 bound to the Avicel column. Thus the CB95 appears to possess intrinsic cellulose-binding activity. The bound CB95, however, appeared in both the H_2O-eluted and SDS-eluted fractions, about half each, but none with high salt (Fig. 3-2). Since the H_2O-eluted CB95 band from Fig 3-1B is a single species of protein (See below), the differential elution property suggested that the conformation of CB95, which failed to be eluted by H_2O, might have changed during the experiment.

To identify CB95, CNBr-generated fragments were separated by SDS-PAGE and microsequenced by Edman degradation. The sequences GNVNPSGRLPLTYPGTTGDI and AYLPGLERFKPIANIQ were each obtained twice, which perfectly matched sequences encoded by the *glu*A gene. *glu*A encodes β-glucosidase-1 during growth and early development, and β-glucosidase-2 during late development (Bush et al., 1994). Since the interspore matrix is produced during late development, the Mr 95,000 band is likely to be β-glucosidase-2. This was substantiated by amino acid composition analysis, which revealed a close match with the conceptual composition predicted after cleavage of the signal peptide after residue 24 (Table 3-1).

Two dimensional gel electrophoresis of the H_2O-eluted fraction revealed a single protein species with an apparent pI of 6.0 (Fig. 3-3A). Western blotting of the same fraction with anti-β-glucosidase mAbs (Mierendorf and Dimond, 1983) yielded an intense reaction (Fig 3-3B lane 1), as expected from the sequence and composition data. The majority of β-glucosidase was found in the interspore matrix compared to the coat (Fig 3-3B, lane ISM, Coats). The appearance of β-glucosidase in the ISM does not seem to vary when the spores were suspended by 50 mM NH_4Ac or H_2, indicating that it is naturally present in the ISM rather than being eluted from the coat during experiment. It is possible that β-glucosidase was not incorporated efficiently into the coat due to the distinctive mechanism of cellulose-binding as compared to SP35 and SP85 discussed in Chapter 2.

Discussion

Application of the Avicel column assay to the interspore matrix proteins identified an intrinsic cellulose-binding activity in a Mr 95,000 protein which was found in both strains of Ax3 and X22 (Figs. 3-1, 3-2). Since this protein comigrated with the SP85 cellulose binding activity isolated from coats as described in Chapter 2, it was of interest to characterize its identity. Peptide sequencing, amino acid composition, western blotting and 2-D gel analysis showed that CB95 was not SP85 but a β-glucosidase encoded by *glu*A gene locus (Fig. 3-3, 3-4; Bush et al., 1994). As discussed in Chapter 2, cellulose-binding proteins are commonly involved in cellulose-degradation. However, cellulose-binding activity is not expected from β-glucosidase, whose substrate is cellobiose (Coston and Loomis, 1969), a soluble cellulose degradation product, suggesting a novel function associated with its cellulose-binding activity.

The mechanism for the noncovalent binding of known CBDs to cellulose is not fully understood. Molecular studies showed that some cellulose-binding activities are dependent on aromatic amino acids, tryptophan and tyrosine in particular, which might directly involve in both hydrophobic and hydrogen bond interactions with cellulose (Reinikainen, et al., 1992; Din et al., 1994). β-glucosidase was eluted from cellulose by H_2O or SDS but not high salt (Fig. 3-1, 3-2), suggesting that the binding is likely dependent on hydrophobic rather than ionic interactions. Since the sequence of β-glucosidase does not show significant homology to the known families of CBD (Bayer et al., 1996), it may contain a novel type of CBD with a novel function.

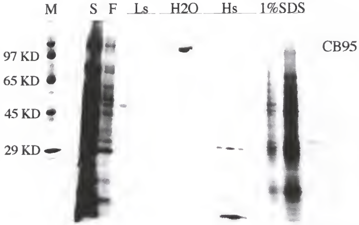
The distinct cellulose-binding mechanism of β-glucosidase, and its enrichment in the ISM separated away from coat cellulose, suggested that the localization of this protein is a regulated process. In conclusion, the ISM may have distinct functions rather than being merely a reservoir of default destination of coat proteins. The cellulose-binding assay with the ISM failed to explore major cellulose-binding coat proteins, probably because these proteins were mostly incorporated into the coat as described in the next Chapter.

Table 3-1. Amino acid composition of the Mr 95,000 protein band and the *glu*A product after removal of the signal peptide sequence.

	Amino acid (%) composition	
Amino acid	*glu*A (25-821)	Mr 95,000 band
Asx	14.4	15.1
Glx	13.1	12.4
His	2.4	1.8
Arg	3.6	4.2
Ala	10.0	10.4
Pro	7.3	9.0
Tyr	6.0	6.5
Val	9.8	9.7
Ile	10.5	9.0
Leu	13.1	11.6
Phe	5.0	4.6
Lys	4.7	5.7

Fig. 3-1. Affinity chromatography of interspore matrix proteins on Avicel. The interspore matrix fraction (S) from strain Ax3 (A) or strain X22 (B) was applied to an Avicel column with a 1 ml bed volume and allowed to flow through (F1, F2) under gravity. Proteins were eluted sequentially with 50 mM NH_4Ac, pH 7.4 (Low salt), H_2O, 1 M NH_4Ac, pH 7.4 (High salt), and 1 % SDS in 50 mM NH_4Ac, pH 7.4 with the presence of 2-ME in each of the solutions. Similar proportions of the loading sample (S), flow through (F1, F2) and successive elution fractions were monitored by SDS-PAGE followed by staining with Coomassie Blue. M, Mr marker.

A) Avicel binding of interspore matrix from Ax3



B) Avicel binding of interspore matrix from X22

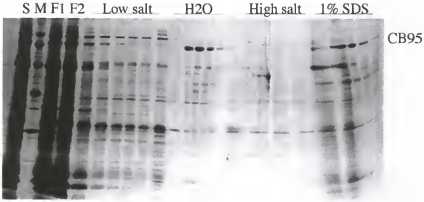


Fig. 3-2. Re-chromatography of the H_2O-eluted fraction from Fig. 3-1B. The H_2O-eluted fractions from Fig. 3-1B were adjusted to 5% 2-ME and 50 mM NH_4Ac with 1 M NH_4Ac, pH 7.4, and reapplied to an Avicel column. The bound proteins were eluted as in Fig. 3-1.

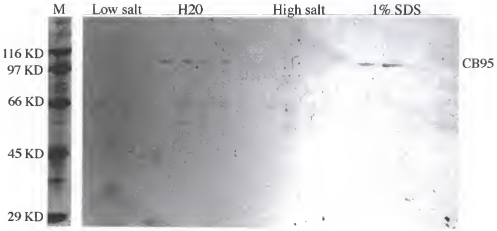
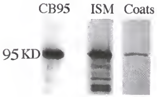

M
Low salt
H20
High salt
1% SDS
116 KD
97 KD
66 KD
45 KD
29 KD
CB95

Fig. 3-3. Electrophoretic analysis of β-glucosidase. (A) 2-D gel analysis of a H_2O-eluted fraction of strain X22 analyzed in Fig. 3-2B. (B) Western blot analysis with anti β-glucosidase monoclonal antibodies. Proteins, approximately equivalent to 10^6 spores, of a H_2O-eluted fraction analyzed in Fig. 3-2B, the interspore matrix and coat proteins of pregerminated spores of strain Ax3, were subjected to Western blotting and probing with a mixture of anti-β-glucosidase mAbs (1A12, 1E11, 5C8 and 4C2). The Mr 95,000 position, at which β-glucosidase-2 migrates, is indicated.

A) 2-D gel analysis of CB95

B) Western blot with anti-glucosidase



CHAPTER 4
A CROSS-LINKING FUNCTION FOR SP85 IN THE SPORE COAT OF *DICTYOSTELIUM DISCOIDEUM*

Introduction

During development of *Dictyostelium*, coat proteins are secreted via prespore vesicles into the extracellular space as an amorphous molecular pool which assembles into distinctive layers with the appearance of cellulose (West and Erdos, 1992). The coincident presence of protein and cellulose in the beginning of coat formation and their adjacent localization in the mature coat suggest that there are interactions between protein and cellulose. Specific molecular interactions are believed to be the common theme in extracellular matrix (ECM) formation, which in vertebrates is largely contributed by structural proteins whose modular domains are involved in multiple interactions with other components (Engvall and Wewer, 1996). Similar to vertebrate ECM proteins, the sequences of seven coat proteins suggest that they are modular consisting of cysteine rich EGF-like and serine /threonine rich mucin-like repeats (West et al., 1996). In the spore coat of *Dictyostelium,* the modular proteins and cellulose may mutually organize each other during coat morphogenesis through direct and indirect interactions.

Earlier *in vitro* studies have identified SP35 and SP85 as two direct cellulose-binding proteins (Chapter 2). The *in vitro* cellulose-binding assays also indicated SP35 and SP85 might anchor other coat proteins, for example SP65, to cellulose like a cross-linker. The sequence of SP85 predicts that it has three motifs, including N-, middle, and C-terminal region, separated by two blocks of TXPP tetrapeptide repeats (Fig. 2-4, 4-1). The C-terminal region may consist of up to five subdomains based on the presence of these domains or subdomains may have distinctive roles besides the cellulose-binding activity of the C-terminal region, which may anchor SP85 into the coat by binding to cellulose.

In this study, the possible cross-linking functions of SP85 observed *in vitro* were tested *in vivo* by using genetic approaches. To investigate roles of the individual domains of SP85, the *psp*B gene locus was either disrupted by gene replacement with a blasticidin selection marker (Adachi et al., 1994) or partially expressed in prespore cells as recombinant proteins. This series of SP85 mutants were compared for possible changes in coat composition, structure and function. The study showed that SP85 was anchored in the coat through its C-terminal domain, which is also responsible for coat incorporation of SP65. Some of the mutants exhibited a decrease in coat density, an increase in coat permeability to a macromolecular tracer and efficiency of spore germination which requires partial degradation of protein and cellulose. These phenotypes of mutants can be interpreted in terms of the cross-linking model. Altogether the *in vivo* results showed that SP85 is essential for both structural and functional integrity of spore coat.

Materials and Methods

pspB Disruption Construct

The disruption vector (pcdBNBsr13) was derived from the *psp*B cDNA plasmid 14E6 Pst73#1 (Mehdy et al., 1983; Powell-Coffman and Firtel, 1994) by insertion of blasticidin resistant (Bsr) marker into *psp*B coding region (Fig. 4-1A). The 14E6 Pst73#1 was cut with NsiI I and BstE II to remove the entire TXPP coding region. The 1.4 kb Bsr marker was derived from pUCBsrdBam (Adachi et al., 1994) by restriction enzyme digestion with Hind III and Xba I. The linear 14E6 Pst73#1 and Bsr marker were purified on 1% a GTG (Seakem) agarose gel with a Qiagen gel extraction kit, and blunt ended with Klenow enzyme. The blunt ended vector was further treated with calf intestine phosphatase to prevent self-ligation with T7 ligase. The ligation mixture was used to transform DH5αMCR cells. Colonies were screened by PCR and restriction enzyme digestion as described in Chapter 2.

Prespore Specific Expression Construct

The expression vectors were modified from pVEIIdATG (Rebstein et al., 1993) in three steps. First, an insert encoding the *cel*A signal sequence (Ramalingam et al., 1992) and a c-myc epitope tag were made by elongation of two annealing oligomers with Taq polymerase. The oligomers were made in the DNA Synthesis Core at University of Florida as follows:

Kpn I Bcl I
5' aggtacctga tca**atg**aaaa ttttaaaaaa ttgtatttta ttaattattt ttggtttatt atCAACTCAA
TTAATTAATG CTGATAG 3'

Sac I Bcl I Bam H I
5' agagctctga tca**tta**ggat cctaaatctt cttctgaaat gagtttttgt tcagatCTAT CAGCATTAAT
TAATTGAGTT G 3'

The capital letters are annealing nucleotides. The bold face **atg** and **tta** represent start and stop codons, respectively. The upper and lower oligomers were designed with Kpn I and Sac I on their 5' end, respectively, to allow directional insertion into the Kpn I and Sac I sites of pVEIIdATG during the construction of pVS vector. The sequence of *cel*A and C-myc coding region was confirmed by DNA sequencing. Second, the pVS was digested with Xho I and Kpn I to remove the discoidin promoter and blunt end ligated with the *cot*B promoter which was contained in the Not I and Mef I fragment from *cot*BGal17 (Fosnaugh and Loomis, 1993), giving rise to pVSB vector. Finally, the expression vectors were constructed by insertion of the Bam H I ended fragments encoding N/C, N and C-terminal fragments of SP85 from the bacteria expression vectors described in Chapter 2, respectively, into the Bgl II site between *Cel*A and c-myc sequence in the pVSB vector (see Fig. 4-1B). Plasmids were amplified in DH5αMCR cells and screened with PCR and restriction enzyme digestion as described in Chapter 2.

Disruption of *psp*B Gene Locus

The disruption vector pcdBNBsr13 was prepared with a Qiagen maxiprep kit. The vector was first cut with XbaI and Hind III (New England). The digestion mixture was treated in a 100 µl volume with 5 units of Bal 31 (Gibco BRL product) in 50 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 600 mM NaCl and 50 µg/ml BSA, at 30 °C for 4 min to remove terminal linker sequences nonhomologous to *psp*B gene. Digestion was monitored by EtBr staining after agarose electrophoresis. The DNA fragments were purified by phenol extraction and ethanol precipitation, and electroporated as above. Aliquots of 200 µl electropolated cells were transferred at clonal density to 96 well plates and allowed to recover for 12-15 h at 22 °C. For colony selection, blasticidin (Sigma) was added to each well to a final concentration of 10 µg/ml. The medium was changed every 3-4 days with HL-5+ (100 µg/ml Ampicilium, 10 µg/ml streptomycin, 5 ng/ml vitamin B12, 2 µg/ml folic acid in HL-5) in the presence of 10 µg/ml blasticidin. After 7-10 days, visible colonies were picked up with a pipette tip and transferred to SM plates for clonal development. Four to five days later, spores ($\sim 10^5$) were picked for screening by western blotting with mAb 16.2.

Protein Expression in Prespore Cells

Plasmids, including pVSBN/C, pVSBC and pVSN, were prepared with a Qiagen maxi preparation column for transformation by electroporation. Cells grown in HL-5 medium were collected at density of 2-5 x 10^6 cells per ml by centrifugation at 1,000 g for 1 minute. The cell pellet was washed once with 50 mM Sucrose, 10 mM KH_2PO4, pH 6.1, and resuspended in the same buffer at 2-4 x 10^7 cells per ml. In a microcentrifuge tube, 0.8 mls of the above cell suspension were mixed with 2-20 μgs of DNA. The mixture was transferred to a pre-cooled 0.4 cm electroporation cuvette and left on ice for 5 minutes. Cells were electroporated in a Bio-Rad gene pulser set at 0.9 KV/3 uF (Adachi et al., 1994). After electroporation, cells were treated immediately with 8 μl 0.1 M $CaCl_2$/0.1 M $MgCl_2$ and left at room temperature for 10 to 15 min. Then they were suspended in 50 ml culture tube with 20 ml HL-5+ and allowed to recover in 100 mm culture dish for 12-15 h at 22 °C. For colony selection, G418 (Sigma) was added to each dish to a final concentration of 10 μg/ml. The medium was changed every 3-4 days with HL-5+ in the presence of 10 μg/ml G418. 10 to 15 days after transformation, visible colonies were transferred with a pipette tip to SM plates for development. After 4-5 days, fruiting bodies, corresponding to 2-10 x 10^5 spores, were picked and screened by Western blotting with antibody 9E10 for expression of the c-my epitope tag.

Coimmunoprecipitation with T7 Tag Affinity Beads

Recombinant proteins, including N/C, N and C-terminal domains of SP85 were purified from inclusion bodies of *E. coli.*, and renatured in 2 M urea with the presence of a glutathione reduction system as described in Chapter 2. The renatured proteins were diluted ten times with T7 tag bind/wash buffer (4.29 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, 0.1% Tween-20, pH 7.3) and incubated with 100 μl T7 tag affinity beads (Novagen) for 30 min at 22 ˚C on a gyrator shaker. The beads were collected and washed twice by centrifugation at 500 g for 3 minutes. The beads loaded with N/C, C or N, respectively, were then incubated with interspore matrix from 2 x10^7 spores of B1 or Ax3 for 1 h at 22 ˚C. After being washed twice with bind/wash buffer, the beads were eluted twice with 100 μl 0.1 M citric acid, pH 2.2 and dryad by vacuum centrifugation. The eluted proteins were analyzed by SDS-PAGE followed by Coomassie blue staining.

Avicel Binding Assay

The N/C, C and N fragments of SP85 were purified and renatured as above. 50 μgs of each fragment was incubated with 1 mg of Avicel in a microcentrifuge tube for 1 h at 22 °C. The Avicel was washed twice with T7 tag bind buffer followed by incubation with the interspore matrix fraction from strain B1 for 1 hour at 22 °C. The Avicel bound proteins were washed twice with the bind buffer and eluted with SDS-PAGE loading buffer for SDS-PAGE followed by Coomassie blue staining.

Electrophoresis, Western Blotting and Peptide Sequencing

Spore coats were prepared from pregermination spores by French press lysis as described in Chapter 2. Proteins from equivalent amounts of coats and interspore matrix were analyzed on 7-15% linear gradient SDS-polyacrylamide gels followed by either Coomassie blue staining or Western blotting. A protein band, corresponding to SP65, was digested with CNBr. Peptide fragments were separated on a Tris-tricine gel and blotted to PVDF membrane for sequencing as described in Chapter 2.

Flow Cytometry of FITC-RCA-I Labeled Spores

Two aliquots of spores (1-2 x 10^6) were collected in TBS (0.05% NaN_3, 10 mM Tris-HCl, 150 mM NaCl, pH 7.4) in a microcentrifuge tube. One aliquot was extracted with 6 M urea/1% (v/v) 2-mercaptoethanol in TBS buffer in a boiling water bath for 3 min. Both aliquots of spores were washed twice with TBS by centrifugation followed by incubation with 2 mg/ml bovine hemoglobin (Sigma) in TBS for 5-10 min. The spores were then incubated with 100 μl of 10 μg/ml FITC-*Ricinus communius* agglutinin I (Vector Laboratories, Ignacio, CA) for 1 h at 22 °C on a shaker. the labeled spores were washed twice with TBS , suspended at 2 x 10^6 ml^{-1} in the same buffer and sonicated on ice at level 5 for 15 s using a microtip on a Branson sonicater. Phase contrast microscope was performed to ensure that this treatment resulted in a single cell suspension. The cell suspension was applied to a FACSort instrument under instruction of Melissa Chen at the ICBR Flow Cytometry lab in University of Florida.

Spore Germination

Spores at 5 x 10^7 ml^{-1} were activated for 30 min either by heat (42 °C), or by 20% DMSO or 8 M urea at 22 °C, respectively in KP buffer (20 mM potassium phosphate, pH 6.5) on a gyrator shaker. Spores were then collected by centrifugation and resuspended in KP buffer at 2 x 10^7 ml-1 for germination at 22 °C. Aliquots (10 µl) of germination solution were taken out every other hour and mixed with 0.1% Calcofluor. Coats and spores were counted on a hemocytometer in an epifluorescence microscope as described in (Aparicio et al., 1990). The percent germination was calculated by dividing the number of coats by the number of coats and spores counted at each time point.

Generation of Antisera

The N and C-terminal fragments of SP85 expressed in *E. coli* were purified from inclusion bodies and renatured in 2 M urea as described in Chapter 2. 30 µgs of each peptide was mixed 1:1 with RIBI adjuvant and injected subcutaneously into each of three mice. An additional identical injection was made after 2 weeks. After an additional 3 weeks, 30 µgs of each peptide in 1:1 mixture with RIBI adjuvant was injected intraperitoneally. 3 days later, the mice were primed for ascites induction by injection of 0.5 ml of Pristane. After 5 days, 2 x 10^6 SP2/0 cells were injected intraperitoneally to induce ascites. Ascites fluid was collected 10-14 days later. The test sera or ascites were used in a dilution of 1:500 for Western blotting.

Results

Mutants of SP85 Were Established in Strain Ax3

To study functions of SP85 in vivo, a series of mutants was established as listed in Table 4-1. To create a SP85 null mutation, a portion of the pspB locus was replaced with a blasticidin resistance marker (Adachi et al., 1994) in strain Ax3 (Fig. 4-1A), resulting in the independent strains B1, B2 and B3. The B strains were identified by failure of fruiting bodies to bind mAb 16.1, which recognizes *mod*B glycosylation on SP85 (Fig. 4-2A). Spore coats from B1 were also probed by Western blotting with mouse antisera NT7 and CT7, generated against the renatured N- and C-terminal regions of SP85 expressed E.coli, respectively (Table 4-1). They were unreactive with CT7 (Fig. 4-3 E, F), suggesting no portion of the C-terminus of SP85 was expressed in B1. The NT7 recognized a weak band in the coat fraction (lane B1 in Fig 4-3D) at the Mr position of 24,000. This is unlikely to represent an SP85 fragment as coding information for only 39 amino acids at the N-terminus of SP85 were present in the disruption construct.

The expected genetic change in strain B1 was confirmed by PCR analysis of genomic DNA using primers P1 and P4 (Fig. 4-1A). These primers amplified a DNA fragment with a length of 2.1 kb compared to 1.65 kb from Ax3 (Fig. 4-2B), consistent with the replacement of the coding DNA with the Bsr locus (Fig. 4-1A). A previous attempt to disrupt *psp*B by transformation of the Thy-1 marker inserted into the EcoR V in the pG219 genomic plasmid (Powell-Coffman and Firtel, 1994), failed probably due to 1) the nonhomologous sequence on the 3' end of the cloned *psp*B and 2) a 550 nucleotide deletion, which had occurred in pG219 as shown in Chapter 2. In order to avoid this potential problem, we treated the linearized transforming DNA fragment with Bal 31 exonuclease to remove linker sequences derived from the plasmid. Initial trials without Bal 31 treatment were not successful, indicating that it is important for both ends of replacing DNA to match those of target DNA.

The sequence of SP85 predicts that it has three motifs, including N-, middle, and C-terminal regions, separated by two blocks of TXPP tetrapeptide repeats, which may serve as spacers (Fig. 2-4, Fig. 4-1). To study the functions of these domains, individual N-terminal, C-terminal and N/C-hybrid fragments of SP85 were expressed coordinately with other coat proteins using the prespore specific expression vector, pVSB. pVSB contained the *cot*B prespore specific promoter, the *cel*A cleavable signal sequence, the c-myc epitope tag at the C-terminus of the coding region, and a G418 resistance marker (Fig. 4-1B). Strains expressing SP85-related polypeptides were named by two letters with the first letter from parental strain, either A (Ax3) or B (B1) followed by N, C or H (N/C hybrid), representing expression of N-, C- and N/C hybrid of SP85, respectively. They were selected by G418 and screened by Western blot analysis of fruiting bodies with mAb 9E10 recognizing the c-myc epitope tag, and mouse antisera NT7 and CT7. The expected polypeptides were detected by mAb 9E10 in coats of AN, AC and AH with apparent Mr values of 25,000, 28,000 and 55,000 similar to the predicted Mr values of 23,249, 25,711 and 50,879, respectively, with N being least abundant (Fig. 4-3B).

Each of these mutant strains produced normal-appearing fruiting bodies in the range of 24-40 h. Spores and coats appeared normal by phase contrast and electron microscopy. However, coats isolated from strains AN, AC, B1 and BC exhibited a reduced density on renografin gradients compared to those of Ax3, AH and BH (Table 4-

1). This indicated that deletion or separate expression of individual domains of SP85 physically altered the coats, and that expression of N/C in strain BH rescued the density decrease caused by deletion of SP85.

SP85 Is Anchored in the Coat by its C-terminal Region

The C-region of SP85 exhibited cellulose-binding activity *in vitro* (Chapter 2), suggesting that it may anchor SP85 into the coat *in vivo*. This hypothesis was tested by a comparison of proteins between coat and the interspore matrix. After differentiation, spores are bathed in a fluid interspore matrix, where proteins which fail to incorporate into the coat during extracellular assembly may accumulate. This suggested a convenient assay for measuring the efficiency of incorporation of secreted proteins into the coat. Sori from each of the mutants were suspended in water and centrifuged to separate spores from the interspore matrix in the supernatant. Coats were isolated from spores after mechanical lysis and purified by density gradient centrifugation. Proteins of the interspore matrix and coats were compared by SDS-PAGE followed by Western blot probing with mAb 9E10, NT7 or CT7 (Fig. 4-3). Based on labeling with mAb 9E10, all of the N/C and C fragments were incorporated into the coat. In contrast the majority of N accumulated in the interspore matrix (compare lanes in Fig. 4-3 A, B). In order to assess the relative levels of the polypeptides to endogenous SP85, parallel blots were probed with NT7 and CT7. Labeling with NT7 or CT7 suggested that the N/C fragment was expressed at a somewhat higher level than SP85 with a similar efficiency of incorporation into the coat relative to the ISM (compare lane AH in Fig 4-3 C, D; E, F). In addition, these antisera detected Mr heterogeneity close to the position of N/C, suggesting that some degradation of N/C occurred at its termini. This is probably because the N/C is not glycosylated normally as it does not express the *mod*B epitope seen by mAb16.1 (data not shown). Other coat proteins not expressing the *mod*B epitope are proteolyticaly sensitive (Gonzales-Yanes et al., 1989). Also the N/C construct is susceptible to proteolysis in *E. coli.* Probing with the CT7 suggested that the C-fragment was expressed at a similar level to that of endogenous SP85 with a similar efficiency of incorporation into the coat (see lane AC in Fig. 4-3E, F). In contrast, the N fragment was expressed at an exceptionally higher level but a much lower level of it was incorporated into the coat than the endogenous SP85 (see lane AN in Fig. 4-3E, F). Binding of the expressed N/C- and C-fragments to the coat did not diminish native SP85 binding (see lanes AH, AC in Fig. 4-3D, F), suggesting a large pool of binding sites. In the absence of SP85, the N/C (see lane BH in Fig. 4-3 A, B, E, F) and the C-fragment (see upper C-band in lane BC of Fig. E, F) also incorporated efficiently into the coat. These results showed that SP85 is bound to the coat via its C-region, probably by virtue of its cellulose-binding activity.

SP65 Is Anchored in the Coat by SP85

To determine if other proteins were anchored in the coat by SP85, coat proteins of strains Ax3 and B1, which lacks SP85, were examined by Coomassie staining after SDS-PAGE (Fig 4-4). A similar pattern of Coomassie blue bands were obtained except that a protein band at the position of SP65 was markedly decreased in B1 (Fig. 4-4, right panel, lane B1). A corresponding increase in a protein band at this position was found in the ISM of B1 in some trials but the background was too high in Fig. 4-4 to determine this. Thus it appears that SP65 was expressed at the cell surface in B1, but failed to incorporate into the coat. SP65 was restored in the coat efficiently by expression of N/C hybrid and partially by C-terminal fragment of SP85 in B1 (Fig. 4-4 lanes BH, BC). This also confirmed that the mis-incorporation of SP65 was indeed caused by deletion of SP85. A corresponding decrease at the expected position of SP85 was not observed on the Coomassie staining gel, but the significance of this is not clear in the absence of knowledge of the relative affinity of SP65 and SP85 for Coomassie blue (Fig. 4-4, lane A3, B1). The expressed C-terminus of SP85 in both AC and BC strains appeared to reach a level comparable to other coat protein bands as shown by Coomassie staining (Fig. 4-4). The N/C hybrid bands on AH and BH are hardly seen on the gel probably because of proteolytic heterogeneity leading to diffuse migration and difficulty of visualization by Coomassie blue, since Western blotting showed that it was expressed (Lanes AH, BH in Fig. 4-3). It had been previously noted that after purification, SP65 bound to Avicel only in FPLC fractions which also contained SP85 or another cellulose-binding protein, SP35. The stoichiometry between SP85 and SP65 from strain X22 in the Avicel bound fraction (Chapter 2), on one or two-dimensional gels of coat proteins was close to 1:1. Taken together, these results suggested that SP65 might be anchored into the coat by direct interaction with SP85, possibly its C-terminal region.

SP65 Binds to the C-terminal Region of SP85

To determine whether there is a direct interaction between SP85 and SP65 and to ask if specific domains of SP85 are involved, binding between these two proteins was investigated *in vitro*. Fragments of SP85 expressed recombinantly in *E. coli*, consisting of the N-domain, C-region, and the N/C-hybrid fragment, were adsorbed by virtue of their T7-epitope tags to an anti-T7 antibody linked to Sepharose. These conjugates were then incubated with the ISM fractions from strains B1 (SP85-) and Ax3 (normal), which appeared to be enriched and deficient in SP65, respectively. Proteins adsorbed to

Sepharose beads were analyzed on SDS-PAGE gel followed by Coomassie blue staining. As shown in figure 4-5A, N and C bound to the Sepharose beads as expected. The N/C band, however, was very weak, possibly due to degradation leading to diffuse migration. Nevertheless, a strong band at the position of SP65 was precipitated from B1 ISM by both the N/C and C-fragments, but not the N-domain, suggesting a direct interaction between SP65 and the C-terminus of SP85 (Fig. 4-5A, right panel). A weak band was recovered from unloaded beads, which appeared to result from non-specific binding to the antibody or Sepharose beads as it was absent from the bead negative control. Lower level of a similar band was precipitated from Ax3 ISM not only by N/C and C but also by N (Fig. 4-5A, left panel). The lower level of SP65 recovered from the ISM of Ax3 was consistent with reduced level of expression of SP65 might be reduced in the ISM by the endogenous SP85 present in the coat.

The C-terminal domain of SP85 consists of four EGF-like repeats and a noncysteine-rich region. It is possible that different subdomains of the C-terminus bind to cellulose and protein, respectively. To find out whether the C-terminus of SP85 can bind to cellulose and SP65 simultaneously, Avicel binding of SP85 and SP65 were analyzed. Fragments of SP85, including the N-domain, C-region and the N/C-hybrid fragment expressed *E. coli*, were incubated with Avicel, which was then incubated with the ISM fractions from strain B1. Proteins adsorbed to Avicel were analyzed by SDS-PAGE followed by Coomassie blue staining. As shown in figure 4-5B, the N/C, C and a low level of N which bound nonspecifically (Chapter 2), were recovered from Avicel. SP65 was found in the Avicel bound fraction only when the Avicel particle contained the N/C- or C-fragments. The simultaneous binding of SP85 to cellulose and SP85 indicated that the two binding activities were spatially separated, which supports the model that SP65 binds to the coat directly via SP85 *in vivo*.

SP65 Is Distinct from other Known Coat Proteins

SP65 was purified by sequential anion and cation exchange chromatography from urea extracts of coats. The SP65 band from SDS-PAGE gels (Fig. 2-2e) was subjected to CNBr digestion, and the resulting peptides separated on a second SDS-PAGE gel. Edman sequencing of 4 bands yielded four novel sequences (Table 4-2). Thus SP65 is not related to other known coat proteins. However, one of the sequences contained a complete C4C Cys-rich motif, which is common to all known *Dictyostelium* coat proteins (West et al., 1996).

Permeability of Spore Coat to a Macromolecular Tracer

It has been postulated that coat proteins contribute to forming a permeability barrier for large molecules such as proteins (Gonzalez-Yanes et al., 1989; Fosnaugh et al., 1994). Coat permeability has been measured previously by fluorescence microscopy of spores labeled with fluorescent RCA-I, a lectin which binds to the Gal/GalNac-rich polysaccharide in the inner layer of spore coat near the plasma membrane (Gonzalez-Yanes et al., 1989). Previously it was shown that selective removal of proteins with urea and a reducing agent (West et al., 1996) greatly enhanced fluorescent labeling of the coat (Gonzalez-Yanes et al., 1989), indicating a barrier function for the proteins. Changes in protein composition might have functional consequences on the permeability barrier of the coat, as suggested by analysis of other coat glycoprotein mutants (Gonzalez-Yanes et al., 1989; Aparicio et al., 1990; Fosnaugh et al., 1994). Here, the level of fluorescence was determined quantitatively by flow cytometry. Representative labeling profiles are shown for B1 spores in Fig. 4-6, which shows that urea extracted spores are more fluorescent than intact spores, which are in turn more fluorescent that spores labeled in the presence of hapten inhibitor β-lactose. Data averaged from three experiments are listed in Table 4-3. Urea extraction led to approximately a doubling of fluorescent labeling of Ax3 spores, from 454 to 865 Units. Labeling in the presence of 200 mM β–lactose yielded only 37 U, showing that the labeling was specific to galuran.

Intact spores of B1 showed a similar level of labeling compared to Ax3, 398 vs. 454 U. This suggests that absence of SP85 had little impact on the permeability barrier of the coat. However, after urea extraction, B1 spores were almost 4 times as fluorescent as the unextracted spores (see Fig. 4-6 for profile), and almost twice as fluorescent as the extracted parental Ax3 strain, 1510 vs. 865. Although it is not known whether this increase reflects a difference in the organization of the remaining proteins, or of the polysaccharide components, it is evident that the absence of SP85 has a substantial effect on coat structure, which is masked by the presence of other coat proteins.

Similar to the deletion of SP85, coexpression of the N-, C- or N/C fragments with SP85 had minor effects on labeling of intact spores, though the reduced labeling of strain AN was reproducible (Table 4-3). However, the urea extracted spores of AC expressing the C-fragment exhibited a high level of labeling, comparable to that seen in the absence of SP85, 1443 vs. 1510 U. Expression of the N-fragment and N/C-hybrid caused a intermediate and minimal increase in labeling, respectively. Thus the C-fragment and possibly the N-fragment, but not the N/C-hybrid, exerted a dominant negative effect on structure, which could be detected after urea extraction.

The effects of expression of the N/C hybrid and C-fragment were enhanced in the absence of endogenous SP85, as labeling of untreated spores of BH and BC strains was almost doubled relative to the parental B strain, 932 and 873 U, respectively. The labeling of each of these strains was also increased after urea extraction, though not quite to that of B1. Since the N/C and C-fragments accumulated in the coat, this suggests that they may interfere with the activity of other components to which they bind, when endogenous SP85 was not present to compete with their action. The expression of the N/C-hybrid in strain B1 failed to reverse the increased level of labeling caused by the deletion of SP85, indicating that missing elements including the 17 TXPP repeats and/or the C4C-repeat between the N- and C- region are also important for this SP85 function.

Spore Germination Efficiency

Spore germination requires degradation of the coat for the amoebae to emerge (Jones et al., 1979; Blume and Ennis, 1991). Emergence is accompanied by the action of cellulases and proteases, which might be rate-limited by substrate accessibility. Thus the time of germination represents an additional, if indirect, measure of coat barrier function from the inside direction. This is consistent with the observation that two separate glycosylation mutants exhibit shortened germination time correlating with increased coat permeability (Gonzalez-Yanes et al., 1989; Aparicio et al., 1990). To determine whether the germination rates are altered, one-day old spores from each strain were activated with heat or by treatment with urea or DMSO at 22 °C, and the time course of germination was determined microscopically. Germinating Ax3 spores emerged with typical sigmoid kinetics with a half-time close to 10 h. (Fig. 4-7A). Unlike wild type strains, not all axenic spores are germination competent at least over the time frame of the experiment. To facilitate kinetic analysis, the results were replotted as percent of maximum germination (Fig. 4-7B). To allow comparisons between experiments, the half time of Ax3 was normalized to 10 h in each trial. The B1 strain germinated with a half-time of 8.4 h and an efficiency of 83%, compared to 10 h and 60% for the parental strain Ax3, respectively (see representative profiles in Fig. 4-7C, data in Table 4-4). The differences were reproducible in multiple trials of three independent *psp*B- strains (data not shown), and were also seen when germination was induced by treatment with either urea or DMSO. Thus the absence of SP85 had an effect which was consistent with reducing barrier function of the coat, possibly facilitating access of degrading enzymes to digest the coat leading to faster and more complete germination. Expression of the N/C-hybrid in strain B1 restored the slower germination half-time and decreased the germination percentage, suggesting that the N/C-hybrid possesses the activities necessary for normal barrier function of SP85 in germination. As expected, expression of the N/C hybrid in Ax3 had no effect on germination (Table 4-4). Expression of the C-fragment in the absence of endogenous SP85 in strain BC resulted in a half-time of germination intermediate between the B1 and Ax3 (Table 4-4), suggesting that the C-fragment only inefficiently rescues the SP85 deficit. Similar to deletion of SP85, expression of either N- or C-fragments in the presence of SP85 increased the rate and efficiency of germination (Table 4-4). Thus it was clear that the fragments exerted a dominant effect with respect to germination.

Discussion

Abnormal expression of SP85 created molecular lesions in the coat. As discussed in Chapter 1, there is evidence that specific molecular interactions are the basis for the formation and function of ECMs. To investigate the significance of protein-cellulose interactions in the spore coat of *Dictyostelium*, we created a series of mutants of a cellulose-binding protein, SP85, by genetic disruption and expression of its discrete structural domains (Table 4-1). Overall, spores and the surrounding coats of the mutants appeared normal under phase contrast microscopy and sections transmission electron microscopy (data not shown). However, using biochemical and functional analyses, we found that the coats derived from these mutants were disorganized, as reflected in the changes of coat properties including density (Table 4-1), protein composition in B1 (Fig. 4-4), permeability to a macromolecular tracer (Fig. 4-6, Table 4-3) and efficiency of spore germination (Fig. 4-7, Table 4-4). These findings indicated that abnormal expression of SP85 reduced intermolecular cross-linking, thereby compromising the barrier function of the coat.

SP85 is anchored in the coat by its C-terminal domain. Sequence analysis of seven cloned coat proteins suggested that these proteins are modular as is typical of matrix proteins (Engel, 1996). The coat proteins consist of interspersed mucin-like repeats, EGF-like repeats and polyanionic and polycationic segments (West et al., 1996). Their modular structures suggested discrete functional roles for each domain. SP85 is distinct from five outer layer coat proteins by its location in the inner/middle layer overlapping with cellulose (West and Erdos, 1992). The cellulose-overlapping location of SP85 correlates with its C-terminal cellulose-binding activity that was not detected in proteins located in the outer layer (Chapter 2). To determine whether the cellulose-binding C-terminal Cys-rich domain of SP85 is essential for coat incorporation of SP85, we examined the destiny of individual N- and C-terminal domains of SP85 using an endogenous prespore specific expression system.

The prespore specific expression was driven by *cot*B, a promoter for another spore coat protein (Fosnaugh and Loomis, 1993). As expected, the transformed*cot*B promoter was properly induced during development because no c-myc tagged proteins were found in vegetative growing cells (data not shown). The efficient incorporation of C-fragment into the coat and the accumulation of N-fragment in the adjacent interspore matrix suggest that they were in the right secretory pathway, probably via prespore vesicles as is typical for other coat proteins (Hemmes et al., 1972; Devine et al., 1983), which provided access to the forming spore coat. Thus the endogenous expression system produced proteins in the correct time and space suitable for coat assembly.

Proteins secreted from prespore cells can either be incorporated into the spore coat or be accumulated in the interspore matrix, the space between spores. Establishing whether a protein was incorporated into the coat can be accomplished by comparing proteins from the coat and the interspore matrix by Western blot analysis. The majority of expressed N/C-hybrid and C-terminal fragments of SP85 were found in the coat extracts while the N-fragment was found in the interspore matrix. This was interpreted to mean that SP85 is normally associated with the coat by virtue of its C-terminal region, based on its cellulose-binding activity which can be demonstrated *in vitro* (Chapter 2).

SP85 is a cross-linker between cellulose and SP65. The incorporation of SP65 in the coat depended on the presence of SP85 because deletion of SP85 caused SP65 to be found in the interspore matrix (Fig 4-4). The interaction between SP65 and SP85 may have started in prespore vesicles, as proposed for the putative PsB complex (Watson et al., 1994), remained in the mature coat in *vivo* and resumed after renaturation following urea extraction of coat in *vitro*. SP85 and SP65 were both found in the flow-through faction of DEAE anionic exchanger column and were coeluted with high salt or high pH from SP cationic exchanger column (Chapter 2). Avicel binding of the partially purified SP fraction, containing both SP65 and SP85, suggested that the level of SP65 in the cellulose-bound fraction depended on SP85 (Fig. 2-3). Co-immunoprecipitation with the T7 tagged N/C-hybrid, N- or C-terminal domains of SP85 suggested that the interaction between SP65 and SP85 were direct and only the C-terminal domain of SP85 interacted with SP65 (Fig. 4-5A). The ability of the C-terminal domain of SP85 to bind simultaneously to cellulose and SP65 (Fig. 4-5B) indicated that separate subdomains on the C-terminus are involved in the two binding events. The interactions of SP85 with SP65 and cellulose observed *in vitro* correlated with the *in vivo* results of deletion or expression of parts of SP85. These data suggest that SP85 functions as a cross-linker between SP65 and cellulose by direct interactions through two separate subdomains on its C-terminus. However, the C- fragment was not able to rescue SP65 as efficiently as the N/C-hybrid in the BC strain *in vivo*. Since no difference in binding of SP65 to N/C or C was observed *in vitro,* this may have been due to a somewhat higher level of expression of the N/C-hybrid suggested by Fig. 4-3C-F, or the ability of the attached N-fragment to enable the presentation of the binding domain on the C-fragment.

Barrier function of the spore coat was altered by abnormal expression of SP85. Proteins in the outer layer cumulatively provide a protective barrier to macromolecules as assayed by labeling with fluorescent RCA-I, a lectin which binds to galactose-polymers in the inner layer of spore coat (West and Erdos, 1990; Fosnaugh et al., 1994). Flow cytometry analysis of FITC–RCA-I labeled spores revealed that the intact spores of each strain exhibited a low level of fluorescence. Removing 85% of coat proteins by urea extraction doubled the level of fluorescence of Ax3 (Table 4-3), indicating that coat proteins are involved in barrier function. Urea extraction of B1 spores yielded a much higher level of fluorescence than the Ax3 spores (Table 4-3), suggesting that the B1 coats were altered, but required unmasking by urea extraction to detect.

Coats from AN and AC also exhibited higher permeability after urea extraction, suggesting that the N or C interfered with the normal function of SP85. Since no major protein was missing in the coat, this indicated that the coat is not merely an additive aggregate of molecules but an ordered structure that can be disrupted by inappropriate incorporation of discrete binding domains isolated from their parent proteins. The higher permeability in AC and AN than AH indicated that the individual domains of N- or C- are more damaging than the N/C hybrid. This dominant negative effect caused by overexpression of N- and C- suggested that individual domains of SP85 were linked functional units as predicted in the cross-linker model. Interaction with SP65 provides an example for how this model is thought to work. Effects on ligands in addition to SP65 are implied, as permeability changes do not correlate in a simple way with SP65 retention. The implied ligand for the N-terminal fragment is not known. It may be a low abundance protein not seen in 1-D SDS-PAGE.

Expression of the N/C-hybrid in BH was not able to reverse the increase of coat permeability caused by deletion of SP85 (Table 4-3). Since the N/C hybrid seemed to be at least as abundant as the level of endogenous SP85 in normal strains (compare lane BH to A3 in Fig. 4-3 D, F), this suggested that the missing elements including the 17 extra TXPP repeats and a Cys-rich repeat were essential for normal function of SP85. The lack of *mod*B glycosylation on the N/C hybrid, possibly due to the missing TXPP repeats which are a potential glycosylation site (Gattie et al., 1994; Seregni et al., 1997; Neumann et al., 1998), might have contributed to the increase of coat permeability as detected in *mod*B mutant strains (Aparicio et al., 1990).

Spore germination was altered in coat protein mutants. Spore germination involves several processes including initial activation or signaling, swelling which is reversible, partial coat degradation and, finally, emergence of the amoeba from the coat. A change in each of these processes may speed up the overall process of germination. An increasingly porous structure is expected to allow better access for proteases and cellulases to degrade their substrates, resulting in a higher germination rate and efficiency. The increased germination rates of B1, AC and AN indicated that the coats from these strains were functionally altered. The cross-linking model invoked to explain the labeling changes with FITC-RCA-I can also explain the germination results because the cross-linking function would be expected to provide a barrier access of secreted cellulase(s) to the cellulose. A possibility raised by the *in vitro* purification data (Chapter 2) is that SP85 may facilitate the targeting of the CelA cellulase to cellulose, but an interaction could not be demonstrated *in vitro* using CelA provided by H. Ennis (data not shown). Expression of the N/C-hybrid in strain BH slowed the germination rate to a level approaching that of Ax3, indicating that the N/C was able to restore functions of SP85. All together, the data showed that the N/C-hybrid was able to bind to cellulose, and to rescue SP65 and spore germination but not the coat permeability barrier property (Fig 4-4, Fig. 4-5, Table 4-4). This suggests that the missing middle domain exerts a cross-linking function required for the external barrier function (tested by FITC-RCA-I) but not the internal barrier function (tested by germination).

Structural proteins are evolutionarily significant. From an evolutionary prospective, modular proteins may have arisen to perform multiple functions as an alternative to protein complexes to provide the same function (Hegyi and Bork, 1997). Modular domains are especially common in extracellular (Engel, 1996) and cytoskeletal structural proteins (Pascual et al., 1997; Puius et al., 1998), and proteins in receptor mediated eukaryotic signaling pathways (Kuriyan and Cowburn, 1997). Two adjacent functional domains in modular proteins, such as the GP115 protein in yeast cell wall (Gatti et al, 1994) and membrane bound thrombomodulin (Tsiang et al., 1992), are separated by linker sequences rich in serine and threonine residues, which may be critical for the proper function of the linked domains. These findings suggest that the two blocks of TXPP repeats in SP85 may function as essential spacers as well. The C-terminus of SP85 contains four C4C repeats with distinctive sequences. Two of them have the same Cys-spacing as fungal type I cellulose-binding domain (Bayer et al., 1996). A similar type of Cys-repeat is also found in the noncatalytic domains of cellulases and hemicellulases of anaerobic fungi and bacteria, which can be found docked to the scaffoldin protein in a multienzyme complex (Fanutti et al., 1995). In fact, Cys-rich domains are typical ligand-binding sites in structural proteins (Mateos et al., 1997; Wu et al., 1996; Rattner et al., 1997) or receptors in signaling pathways (Sappington and Raikhel, 1998). It is possible that one or two of the C4C repeats of SP85 are important for the binding to cellulose while others take part in binding to SP65 and possibly other proteins. The sequence of the N-terminal domain of SP85 is distinct from other coat proteins because of the presence of numerous hydrophobic residues, suggesting tertiary folding. Peptide sequencing of SP65 showed that it is a novel protein with at least one C4C repeat common to all other known coat proteins (Table 2, West et al., 1996), suggesting it also has a modular nature.

It can be concluded that SP85, as a cellulose-binding coat protein, has multiple functions that are structurally separated on the modular domains predicted from its inferred protein sequence. The proper function of these discrete domains of SP85 is essential for the structural and functional integrity of spore coat.

Table 4-1. Cell strains and antibodies used in this study, and coat density of each strain.

Strain	Parental strain	Coat density	SP85-related polypeptides expressed	Cognate antibodies
Ax3	NC-4	1.258	SP85	anti-N, anti-C, mAb 16.1
HW70 (B1)	Ax3	1.251	none	none
HW71 (B2)	Ax3	n.d.	none	none
HW72 (B3)	Ax3	n.d.	none	none
HW60 (AH)	Ax3	1.261	SP85 N/C-hybrid	NT7, CT7, mAb 16.1 NT7, CT7, mAb 9E10
HW61 (AC)	Ax3	1.251	SP85 C-fragment	NT7, CT7, mAb 16.1 CT7, mAb 9E10
HW62 (AN)	Ax3	1.239	SP85 N-fragment	NT7, CT7, mAb 16.1 NT7, mAb 9E10
HW63 (BH)	HW70	1.259	N/C-hybrid	NT7, CT7, mAb 9E10
HW64 (BC)	HW70	1.248	C-fragment	CT7, mAb 9E10

n.d. = not determined

Table 4-2. Edman sequencing of internal peptides of SP65. Four novel sequences were obtained by Edman sequencing the peptide fragments of SP65 generated by CNBr. The sequence in the first column has a C4C repeat similar to those found in other coat proteins.

1	SYDACYNVVCPSNYQCRAE(A)GDQAYCVEHH
2	VRGNSGCLYNHDGI
3	VRGNPTCLRNH(N)D(H)G(D)I(G)S(I)S(I)H(S)XXP
4	IALVSA

Table 4-3. Spore coat permeability tested by labeling with FITC-RCA-I. Spores from each strain were treated with or without 6 M urea/β-ME and labeled with FITC-RCA-I, which specifically binds to the galuran polymer in the inner layer of spore coat. The labeled spores were analyzed by flow cytometry to obtain a fluorescence distribution spectrum plotting forward scattering against fluorescence. A window was drawn around the primary population of single cells, and the mean fluorescence represented by an arbitrary unit was calculated from this group. The number of units were normalized to urea-extracted B1 spores to allow comparison between experiments, since no internal standard was included for instrument calibration.

Strain	Mean fluorescence		
	Non-extracted	Urea-extracted	urea-extracted + β-lactose
Ax3	454 (375, 521, 485)	865 (952, 698, 945)	37
HW70 (B1)	398 (398, 396, 398)	1510 (1510, 1510, 1510)	44
HW62 (AH)	385 (532, 156, 467)	1030 (1369, 656, 1060)	
HW61 (AC)	548 (558, 562, 524)	1440 (1736, 1052, 1540)	
HW60 (AN)	331 (476, 187, 331)	1140 (1369, 910, 1140)	
HW65 (BH)	932 (-, 1364, 500)	1360 (-, 1218, 1510)	
HW66 (BC)	873 (-, 823, 923)	1210 (-, 1073, 1340)	

Numbers in parenthesis are original normalized values from experiments A, B and C reading from left to right.

- indicates not done.

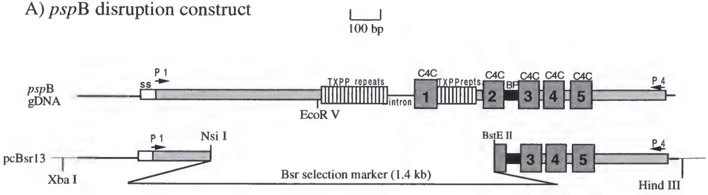
Table 4-4. Germination kinetics of mutant spores. One day old spores from each strain were activated by heat, urea or DMSO for germination. Spores and germinated coats were visualized by Calcoflour-induced fluorescence and counted under the fluorescent microscope. The germination half-time was derived from the plot of time-course germination as in Fig. 4-6 at the point of half-maximum germination. Half-time values are normalized relevance to Ax3, which was set at 10 h to permit comparison between experiments.

	Heat activation		Urea activation		DMSO activation	
	half (h)	Max. (%)	half (h)	Max. (%)	half (h)	Max. (%)
Strain						
Ax3	10 (10, 10, 10)	60 (57, 60, 62)	10	51	10	46
B1	8.4 (8.9, 8.9, 7.5)	79 (71, 81, 86)	8.6	63	7.8	70
AH	9.9 (9.8, -, 10.0)	40 (36, -, 43)				
AC	8.1 (8.8, -, 7.3)	80 (90, -, 70)				
AN	8.3 (8.6, 8.9, 7.5)	77 (70, 72, 88)				
BH	9.7 (8.6, 10.8, -)	63 (63, -, 62)				
BC	9.1 (8.9, 9.2, -)	82 (74, -, 90)				

Numbers in parenthesis are original data from experiments A, B and C, reading from left to right.

- Indicates not done.

Figure 4-1. Plasmid constructs. A). The disruption plasmid pcdBNBsr was constructed from the *psp*B cDNA plasmid, 14E6 Pst#1, in which the 526 bp Nsi I and BstE II fragment of *psp*B was replaced with the 1.4 kb blasticidin resistant marker. B). The prespore specific expression plasmid pVSB was modified from the pVEIIdATG vector by insertion of *cel*A signal and c-myc epitope coding sequence in the Kpn I and Sac I sites and replacing the discoidin promoter with the prespore specific *cot*B promoter. Blunt end ligation of Mfe I and Not I fragment of *cot*B promoter with the Xho I and Kpn I site in pVEIIdATG destroyed all four restriction sites (see Methods). The partial cDNA sequences of SP85 shown were inserted into the Bgl II site between *cel*A and c-myc, such that the coding region of the N/C, C- and N-fragments was in frame with the c-myc epitope on the C-terminus and the cleavable *cel*A signal peptide at the N-terminus.


A) *psp*B disruption construct

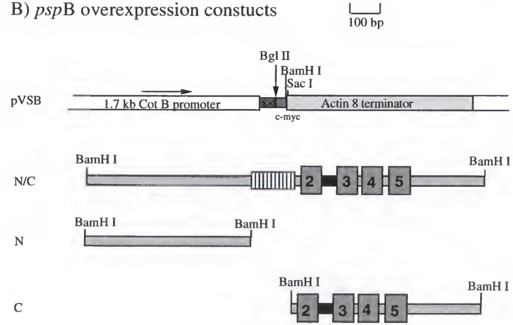
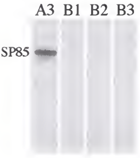

B) *psp*B overexpression constucts

Figure 4-2. Screening of *psp*B disruption strains with Western blot (A) and PCR of genomic DNA (B). A). Spores (5 x 10^5) from individual colonies, B1, B2 and B3 were screened by Western blotting with mAb 16.2. Positive signal of SP85 was detected only in the parental Ax3 strain but not the B strains. B). Genomic DNA from strain Ax3 and B1 were used as templates for PCR with primer P1 and P4 as indicated in Fig 4-1A. PCR products were compared by EtBr staining after separation on a 1% agarose gel. The longer PCR product in strain B1was caused by the insertion of Bsr selection marker in the *psp*B coding region as shown in Fig. 4-1.

A). Western blot with mAb 16.2



B). PCR from gDNA

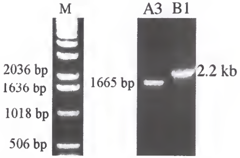


Figure 4-3. Protein composition analysis of mutant coats on Western blots. Proteins equivalent to 5 x 10^5 spores from interspore matrix (A, C, E) and spores (B, D, F) from each strain were analyzed by Western blot with mAb 9E10 (A, B), mouse antisera NT7 (C, D) and CT7 (E, F). M, Mr marker. Positions of key proteins are indicated. The N/C and C-fragments of SP85 were expressed at similar level as the endogenous SP85, and were mostly found in the coat fraction (see lane AH in C, D; AH, AC in E, F). The N-fragment, however, was expressed at higher level than the endogenous SP85, but much lower level was incorporated into the coat (see lane AN in C, D).

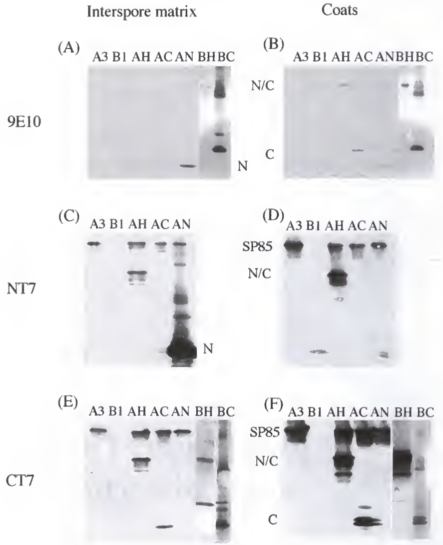
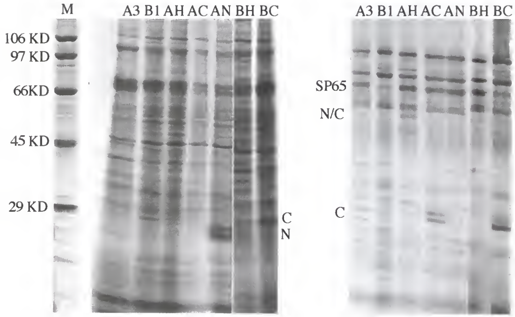

Interspore matrix
Coats
(A)
A3 B1 AH AC AN BH BC
N
(B)
A3 B1 AH AC AN BH BC
N/C
C
9E10
(C)
A3 B1 AH AC AN
N
(D)
A3 B1 AH AC AN
SP85
N/C
NT7
(E)
A3 B1 AH AC AN BH BC
(F)
A3 B1 AH AC AN BH BC
SP85
N/C
C
CT7

Figure 4-4. Protein composition analysis on Coomassie stained gel. Proteins equivalent to 1 X10^7 spores from the interspore matrix and spore coats of various strains were analyzed on 7-20% acrylamide SDS-PAGE gel followed by Coomassie blue staining. M, Mr marker. Note, SP65 was depleted in the coats from B1 strain (lane B1), and was rescued by the expression of the N/C or the C-terminus of SP85 in strain B1 (lanes BH, BC).



Interspore matrix

Coats

Figure 4-5. Binding of SP65 to SP85 *in vitro*. A). Anti-T7 tag conjugated Sepharose beads were used to purify T7-tagged recombinant proteins of SP85 from *E. coli* (Fig. 2-8). The interspore matrix from Ax3 and B1 was mixed with beads that had been pre-incubated with N/C, C or N of SP85, with unloaded beads or with no beads. B). The interspore matrix from B1 was incubated with 1 mg of Avicel that had been pre-incubated with buffer, N/C, C or N of SP85. Bound proteins were washed and eluted with SDS-PAGE loading buffer and 5% β-ME for analysis on a 7-20% acrylamide gel followed by Coomassie blue staining. Nb, no beads; Bd, beads only; H, N/C hybrid; M, Mr marker.

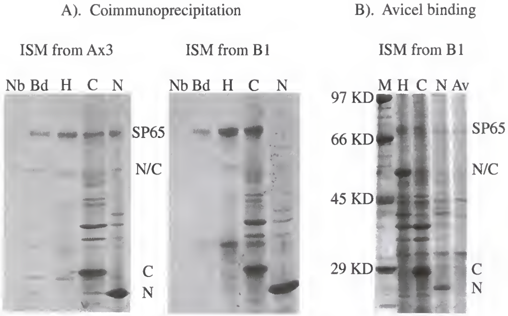

A). Coimmunoprecipitation
B). Avicel binding
ISM from Ax3
ISM from B1
ISM from B1
Nb Bd H C N
Nb Bd H C N
M H C N Av
97 KD
66 KD
45 KD
29 KD
SP65
N/C
C
N
SP65
N/C
C
N

Figure 4-6. Fluorescent labeling of B1 spores with FITC-RCA-I. B1 spores, untreated (B1, B1 CTL) or preextracted with urea (B1 U), were labeled with FITC-RCA-I in the absence (B1, B1 U) or presence of the hapten inhibitor β-lactose (B1 CTL). The fluorescently labeled spores were analyzed by flow cytometry. Level of fluorescence was increased by urea extraction (B1 U), which removes the majority of coat proteins, and reduced by β-lactose (B1 CTL), which competes for binding of RCA-I to the galuran in the inner layer of spore coat.

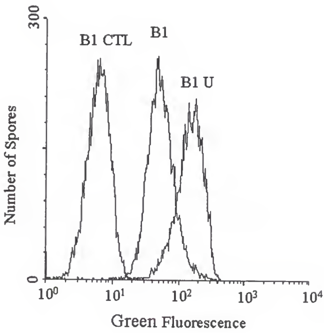
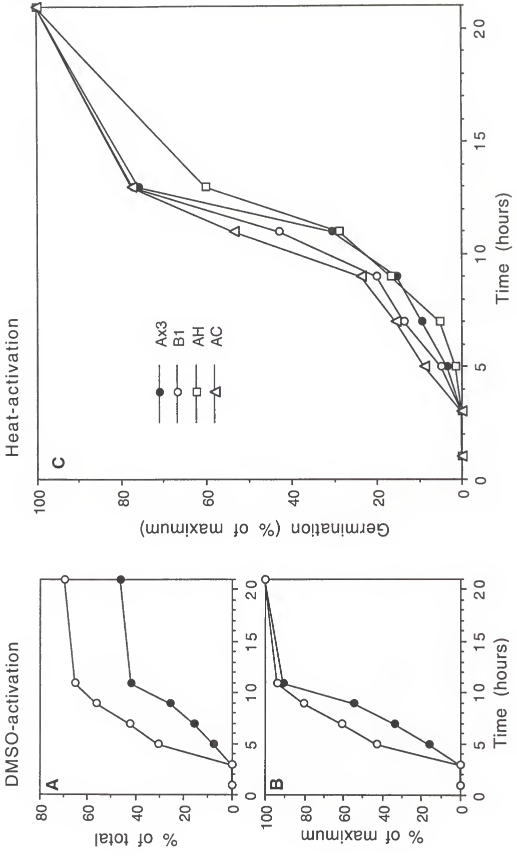

300
0
Number of Spores
B1 CTL
B1
B1 U
10^0
10^1
10^2
10^3
10^4
Green Fluorescence

Figure 4-7. Spore germination time course. Spores (1 day old) from each strains were activated with heat or DMSO for 30 minutes and placed on a gyrator shaker for germination. Spores and coats visualized by Calcoflour-induced fluorescence were counted in the fluorescent microscope. The percent germination was calculated by dividing the number of coats by the total number of coats and spores.


DMSO-activation
A
% of total
80
60
40
20
0
B
% of maximum
100
80
60
40
20
0
0
5
10
15
20
Time (hours)
Heat-activation
C
Germination (% of maximum)
100
80
60
40
20
0
0
5
10
15
20
Time (hours)
Ax3
B1
AH
AC

CHAPTER 5
SUMMARY AND CONCLUSION

Extracellular matrix (ECM) is universally present on the surface of both prokaryotic and eukaryotic cells as an essential part of a living organism. The structure and components of ECM varies among different organisms and their tissues. The common theme is that the diverse array of molecules interact with each other specifically to form an ordered functional structure. The study of the specific molecular interactions was slowed by the complexity of tissue types, irreversible covalent interactions between components and the effort required to perform genetic analysis in complex systems such as animal. In the past few decades, *Dictyostelium* has been recognized as a favorable organism to study cell differentiation and morphogenesis due to the simplicity of its life cycle and body forms compared with other phyla (Gilbert, 1994). For the same reason, the spore coat of *Dictyostelium* offers a special opportunity for understanding protein-polysaccharide interactions in the formation and function of ECM.

The spore coat is formed as a rigid extracellular wall around each spore during development of *Dictyostelium*. As the vegetative growing cell differentiate into either prespore or prestalk cells, coat proteins and a small percent of galuran are synthesized and stored in the prespore vesicles (Hemmes et al., 1972; Devine et al., 1983). At later development, the prespore vesicles fuse with the plasma membrane and the future coat proteins and galuran are exocytosed as an amorphous pool that is shared between neighboring cells (West and Erdos, 1992). Synthesis of cellulose, the major polysaccharide of the coat, from the plasma membrane (Blanton and Northcort, 1990;

Blanton, 1993) coincides with the assembly of coat proteins into discrete double layers, 30 nm each, with about 150 nm thick cellulose in between (Hemmes et al., 1972; West and Erdos, 1992). Epitopes on a PsB protein complex (Watson et al., 1994) observed in prespore cells were located either in the outer or inner layer, indicating assembly might occur extracellularly. However, proteins from the outer and inner layers may still interact through the cellulose layer, since coat proteins, with an average length of 500 amino acids, are rich in sequences predicting rod-like structures (West et al., 1996). It would take about 300 amino acids to extend through the cellulose layer, counting 2.5 Å per residue (Jentoft, 1990). Unlike proteins in the ECM of bacteria (Hills, 1973; Catt et al., 1978; Aronson et al., 1992) and animal systems (Leblond and Inoue, 1989), which can self-assemble to form an insoluble structure, the coat proteins of *Dictyostelium* failed to precipitate in the absence of cellulose. We proposed that protein-cellulose interactions, which are hard to investigate in the plant cell wall due to the limited amount of uncharacterized protein, are involved in the formation of spore coat.

In the first part of this project, an *in vitro* cellulose-binding assay was used to search for proteins that have intrinsic cellulose-binding activity. Two coat proteins, SP35 and SP85, encoded by the *psv*A and *psp*B genes, respectively, were found to bind cellulose more efficiently than other coat proteins. The binding was specific to cellulose, consistent with it being the major polysaccharide of the coat, but not with Sephadex or Sepharose. After partial purification with anion and cation exchange columns, only SP35 and SP85 were still able to bind cellulose as a single major band as revealed on a Coomassie stained gel. These observations suggest that other coat proteins might bind to cellulose indirectly by interacting with SP35 or SP85. This is supported by the finding that SP65 and a germination specific cellulase, copurified with SP85 in some HPLC fractions. The presence of SP85 increased the binding of SP65 to cellulose. These results predict that SP85 might have a cross-linking role between cellulose and other proteins. The cellulose-binding activity of SP85 was confirmed and localized to the C-terminal region by expression of portions of *psp*B cDNA in *E coli*.

The interspore matrix (ISM) contains part of the coat proteins that failed to incorporate into the coat but that remain in a soluble native form suitable for study without prior process of denaturation and renaturation. As shown in Chapter 3, the β-glucosidase-2 from the ISM was found to bind cellulose in a H_2O-eluting fashion. This is distinct from SP35 and SP85, which were not eluted by H_2O or high salt but required urea or SDS, indicating different mechanisms in cellulose-binding were used by proteins in the ISM and coats. The elution of β-glucosidase-2 from cellulose with H_2O *in vitro* and its in *vivo* location in ISM suggest that the microenvironment within the spore coat has low ionic strength, which may provide a mechanism for regulation of coat assembly.

In chapter 4, the cross-linking function of SP85 observed *in vitro* was tested *in vivo* using gene disruption and recombinant expression approaches. Mutant strains that contained deletions of or abnormal expression of SP85 produced spores and coats that looked normal under microscopical examination, which suggest that SP85 is not essential for the coat assembly. However, as discussed in Chapter 2, other cellulose-binding proteins, such as SP35, might mediate coat assembly in the absence of SP85. In fact, redundancy is a common strategy in biological systems to ensure basic functions. In the spore coat of *Dictyostelium*, this redundancy may be is reflected in the sequence similarity of its coat proteins (West et al., 1996). The cellulose-binding coat proteins may also function in a redundant fashion. Nevertheless, the data derived from SP85 revealed that specific molecular interactions are essential for the structural and functional integrity of ECM.

The deletion of SP85 was caused by partial replacement of the *psp*B coding region with a blasticidin selection marker. This SP85 deletion resulted in the failure of incorporation of SP65 in coat. Expression of N-, C-terminal and N/C-hybrid fragment of SP85 in appropriate time and space as normal coat proteins showed that the C-terminal Cys-rich region of SP85 is responsible for coat incorporation of the whole protein. Expression of the C-terminal region and N/C fragment in the SP85 null mutants rescued SP65 in the coat, which is consistent with the ability of the C-terminal region to bind SP65 *in vitro*. Altogether, the data showed that the C-terminal region of SP85 anchored SP85 itself and SP65 in the coat, presumably through direct cellulose-binding and protein-protein interaction as depicted in the diagram of Fig. 5-1.

Although spores and their coats derived from the SP85 mutant strains appeared normal under microscopy, their physical and functional properties were changed. A decrease in coat density was observed in strains missing SP85 or expressing the individual N- or the C-terminal regions of SP85. Except for the missing SP65 in the SP85 null strain, the protein composition of each mutant strain appeared similar to the normal stain on Coomassie blue stained gels. Thus the dominant negative effects of N- and C-fragments may have resulted from loose molecule packing rather than mis-incorporation of components. Analysis of coat barrier function with a macromolecular probe, FITC-RCA-I, showed that the coats from each strains exhibited higher permeability than Ax3 that was unmasked only by removing the coat proteins, indicating that the galuran in the remaining polysaccharide skeleton was possibly more exposed. The increased coat permeability was mostly correlated with an increase in rate and efficiency of spore germination which involves partial coat degradation by cellulases and proteases. However, expression of the N/C-hybrid in the SP85 null strain restored the normal germination rate but failed to reduce the coat permeability to a normal level. It is possible that the negative *mod*B glycosylation, presumably on the missing 17 TXPP repeats (Gattie et al., 1994; Seregni et al., 1997; Neumann et al., 1998) or the deleted C4C repeat in N/C-hybrid had major impact on coat permeability but less on spore germination. The negative effects caused by the expression of the N-terminus in the presence of endogenous SP85 suggested that the N-terminus may be involved in the interactions with one or more other coat components (Fig 5-1).

Although separate barrier functions may be mediated at the external face and internal regions of the cellulose matrix, SP85 can be envisioned to contribute to both by virtue of its two tandem TXPP tetrapeptide repeat clusters, which connect and would allow distant spacing of the N and C binding domains. Altogether, the *in vitro* and the *in vivo* data together indicated that SP85 contributed in an essential fashion to a barrier function of the coat as a cross-linker, by virtue of individual domains mediating specific protein-protein and protein-cellulose interactions. Specifically, this study showed that the interactions of SP85-cellulose and SP65-SP85 are necessary for the incorporation of SP85 and SP65, respectively, into the spore coat. It also indicated that there are specific interactions that are required for the coat function as suggested by the negative effects caused by the expression of the N-terminus or deletion of the middle domain of SP85.

In conclusion, the investigation about cellulose-binding proteins in the spore coat of *Dictyostelium Discoideum* suggested that the spore coat is not a nonspecific aggregate of molecules but rather an ordered structure formed through specific molecular interactions. This type of specific molecular interactions may also apply to the ECMs of vertebrates as discussed in Chapter 1.

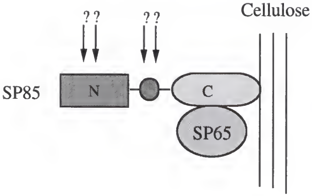


Figure 5-1. Model of SP85 as a cross-linker. The deduced amino acid sequence of SP85 predicts that it is a modular protein consisting of a N-terminal (N), a middle (M) and a C-terminal (C) domain, separated by two blocks of tandem TXPP tetrapeptide repeats. SP85 is anchored in the coat by binding to cellulose with its C-terminal domain, which also links SP65 in the coat, probably through a direct interaction. An unknown ligand for the N-terminus is suggested by its dominant negative effects on coat barrier functions. The middle domain contributes to the permeability function of spore coat.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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